REMARKS

Applicants respectfully request reconsideration of the rejections set forth in the Final Office Action mailed on June 30, 2008.

Claims 1, 5, 6, and 12-37 had been pending and claims 1, 5, and 6 were examined. Claims 2-4 and 7-11 were previously cancelled without prejudice or disclaimer. Pending entry of this amendment, claim 1 has been amended and claim 5 has been cancelled without prejudice or disclaimer. Thus, with this amendment, claims 1 and 6 are under consideration.

Claim 1 has been amended to recite "measuring production of the protein or salt thereof and the binding activity of the protein or salt thereof to the polynucleotide." Support is found throughout the specification, for example, at least at page 30, line 21 to page 31, line 10, and page 31, line 19 to page 32, line 5.

Claim 1 has been amended to recite "comparing production of the protein or salt thereof and the binding activity of the protein or salt thereof when the cell is cultivated in the absence of the test compound and when the cell is cultivated in the presence of the test compound." Support is found throughout the specification, for example, at least at page 30, line 21 to page 31, line 6.

Claim 1 has also been amended to recite a step of "selecting the compound of step (f) that decreases the level of at least one selected from urinary albumin excretion, TGF-β1, ICAM-1, tissue factor, PDGF-B, fibronectin, and α1 (I) collagen." Support is found throughout the specification, for example, at least at the Examples at page 65, line 16 to page 76, line 6.

Accordingly, the amendments are fully supported by the specification as filed. Upon entry of the present amendments, claims 1 and 6 will be under consideration.

Applicants address below each issue raised in the Final Office Action of June 30, 2008.

Preliminary Matters

Information Disclosure Statement

The Examiner contends that "no signature is present on the 1 September [Information Disclosure Statement] submission." Applicants herewith submit a new Information Disclosure Statement citing two of the three documents of the September 1, 2006, Information Disclosure Statement. Applicants note that one of those documents, H.D. Rupprecht et al., "Expression of the Transcriptional Regulator Egr-1 in Experimental Glomerulonephritis: Requirement for Mesangial Cell Proliferation," Kidney International, 51:694-702 (1997) has already been cited by the Examiner. Specifically, that document was cited by the Examiner in the Office Action of October 30, 2007.

Applicants respectfully request that the Examiner consider all of the documents cited in the attached Information Disclosure Statement and then indicate that they have been considered by initialing and returning the attached SB/08 to applicants.

Withdrawn Objections and Rejections

Applicants note with appreciation that the Examiner has withdrawn the objection to claim 5, the rejection of claims 1, 5, and 6 under 35 U.S.C. §§ 112/101, the rejection of claims 1, 5, and 6, under 35 U.S.C. § 112, first paragraph (written description), and the rejection of claims 1, 5, and 6 under 35 U.S.C. § 102(b).

Claim Rejections

I. Rejection of claims 1, 5, and 6 under 35 U.S.C. § 112, First Paragraph

Claims 1, 5, and 6 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Examiner contends that "it would require undue experimentation to practice the invention claimed." Action at page 9.

Regarding the nature of the invention and breadth of the claims, the Examiner stated that "[b]ecause [claim 1] does not interrelate the immobilizing and contacting steps with "measuring a binding activity" the measured binding activity could be the binding of anything to the protein (e.g., antibodies, small molecules, etc.) in addition to the binding of the immobilized polynucleotide implied by the preceding steps." Action at page 4.

The Examiner alleged that the instant art is unpredictable and contented that "the claims are directed to the polypeptide of the claims as a marker of efficacy in the prevention or treatment of any renal disease." Action at page 5. According to the Examiner, "the art teaches that before a putative biomarker can be used as a surrogate endpoint it must be validated as such." *Id.* The Examiner also stated that "one cannot assume that an agent having the capacity to modulate expression of a polypeptide comprising SEQ ID NO: 2 in any given cell type will be capable of modulating expression of the polypeptide in cell types relevant to renal disease." *Id.* at page 6.

The Examiner also urged that "there does not appear to be any evidence presented demonstrating that an agent that alters the expression or function of Egr-1 produces a therapeutic effect in a disease model." Action at page 7. Furthermore, the Examiner noted that the Egr-1 RNA measured in the examples "is most likely regulated at the expression from the endogenous gene" and that "there is no evidence that expression of a protein comprising SEQ ID NO: 2 can be used as a marker for an agent capable of preventing or treating renal disease independent of the endogenous Egr-1 gene regulatory elements." *Id.* at pages 7-8. The Examiner also stated that "the application disclosure does not extend beyond expression of mRNA from the endogenous Egr-1 gene in kidney cells and prophetic statements limited to binding of Egr-1 protein to nucleic acids." *Id.* at page 10.

The Examiner concluded that "even if the invention were enabled for identifying a prophylactic or therapeutic substance for renal disease by measuring expression of an endogenous gene encoding SEQ ID NO: 2 to DNA, extending the teachings of the specification such that the method could be practiced as broadly as claimed would require undue experimentation." Action at page 9.

Applicants respectfully traverse. Contrary to the Examiner's contention, "immobilizing on a solid phase a polynucleotide to which the protein or salt thereof is capable of binding" and "contacting the solid phase with the protein or salt thereof and an antibody against the protein or salt thereof" are related to step (d). Step (d) recites "measuring . . . **the** binding activity of the protein or salt thereof to the polynucleotide," and therefore, relates to the binding of the immobilized polynucleotide.

In addition, the application disclosure does extend beyond mRNA from the endogenous Egr-1 gene in kidney cells. The specification clearly teaches that a nucleic acid encoding a protein comprising the amino acid sequence of SEQ ID NO:2 can be introduced into a cell and expressed. For example, the specification provides that "the protein or salts thereof of the present invention can also be prepared by cultivating a transformant comprising the DNA encoding the protein of the present invention, and subsequently separating and purifying the protein or salts thereof from the resultant culture." Specification at page 17, lines 11-14. Furthermore, Experimental Example 5 shows experiments using cells that overexpress Egr-1. Specifically, Experimental Example 5 states that the "transfection of the Egr-1 expression plasmid increases the Egr-1 protein expression and also the mRNAexpression levels of the renal fibrosis-related genes such as tissue factor, fibronectin, and intercellular adhesion molecule (ICAM)-l." Specification at page 73, lines 17-20. The specification further provides exemplary

host cells, vector, promoters, and signal sequences for generating transformants that are "capable of producing a protein or salt thereof comprising the amino acid sequence of SEQ ID NO:2." *See* specification at page 18, line 23, to page 23, line 16. The exemplary host cells include, for example, bacteria, yeast, and animal cells. *See* specification at page 21, lines 22-23. The specification also describes cultivation of such transformants at page 23, line 17, to page 25, line 16. Thus, the specification describes how to make and use a variety of host cells that overexpress Egr-1.

Moreover, the specification does show to one of skill in the art that production or binding of Egr-1 is a valid measurement for determining whether a compound is a therapeutic substance for a renal disease, and that a nucleic acid encoding a protein comprising SEQ ID NO:2 can indeed be used to screen for such compounds. For example, Experimental Example 5 demonstrated that "transfection of the Egr-1 expression plasmid [in HEK-293 cells] increases . . . the mRNA expression levels of the renal fibrosis-related genes such as tissue factor, fibronectin, and intracellular adhesion molecule (ICAM)-1." Specification at page 73, lines 17-20. Example 1 then demonstrated that Egr-1 antisense oligodeoxynucleotides suppressed Egr-1 protein expression and as a result, suppressed expression of tissue factor mRNA in bFGF-stimulated rat glomerular mesangial cells. *See* specification at page 74, line 13, to page 76, line 6. Thus, these examples demonstrated a correlation between Egr-1 and renal fibrosis-related genes and that suppression of Egr-1 leads to suppression of renal fibrosis-related genes. Other examples in the specification also show a correlation of Egr-1 levels with urinary albumin excretion (Experimental Examples 1, 2 and 4), transforming growth factor-β1 (TGF-β1) (Experimental Examples 1 and 2), fibronectin (Experimental Example 2), PDGF-B (Experimental Example 2), and α1 (I) collagen (Experimental Example 2). As discussed in detail below, these factors are

well recognized in the art as being associated with renal diseases. Therefore, the specification demonstrates that modulation of Egr-1 modulates renal diseases and is not a mere theoretical possibility.

Specifically, the following representative references illustrate the state of the art regarding urinary albumin excretion, TGF-β, ICAM-1, tissue factor, and several fibrosis-related genes and their relevance to kidney diseases known at the time of the earliest filing date of the instant application (January 10, 2002):

- Parving "Microalbuminuria in essential hypertension and diabetes mellitus" *Journal of Hypertension Suppl.* 14(2):S89-93 (1996);
- Rivarola et al. "Transforming growth factor beta activity in urine of patients with type 2 diabetes and diabetic nephropathy" *Braz J Med Biol Res* 32(12): 1525-1528 (1999);
- Dal Canton "Adhesion molecules in renal disease" *Kidney International* 48:1687-1696 (1995);
- McClusky "Tissue factor in Crescentic Glomerulonephritis" *American Journal of Pathology* 150(3):787-792 (1997); and
- Eddy "Molecular insights into renal interstitial fibrosis" *Journal of the American Society of Nephrology* 7:2495-2508 (1996).

At the time of the earliest filing date of the instant application, it was known that "there is evidence that macrophage-derived tissue factor is responsible for glomerular fibrin deposition in human glomerulonephritis." McClusky "Tissue factor in Crescentic Glomerulonephritis" *American Journal of Pathology* 150(3):787-792 (1997) at page 789. In addition, "antibodies to tissue factor reduced glomular tissue factor activation, largely prevented fibrin deposits in Bowman's space, and protected against renal function impairment in rabbits developing anti-[glomerular basement membrane] nephritis." *Id.* at page 788. Thus, a decrease in the level of tissue factor would indicate treatment of a renal disease. Indeed, Experimental Example 5 shows treatment of glomerular mesangial cells with Egr-1 antisense oligodeoxynucleotides when they

decreased tissue factor mRNA compared to controls. Accordingly, reduction in tissue factor showed a therapeutic effect.

It was also known that upregulation of ICAM-1 occurred in a number of kidney diseases, including "classic" nephrotoxic serum nephritis, murine lupus nephritis, focal glomerulonephritis, human lupus nephritis, membranous nephropathy, "active" nephritis, acute and chronic rejection, and hemodialysis. *See* Dal Canton "Adhesion molecules in renal disease" *Kidney International* 48:1687-1696 (1995) at page 1690, Table 2. Thus, one skilled in the art would expect that a reduction in ICAM-1 would have a therapeutic effect against a renal disease. The instant specification does show upregulation of ICAM-1 mRNA and Egr-1 protein in HEK-293 cells transfected with a human Egr-1 expression plasmid (Experimental Example 5), indicating that a compound that decreases the level or binding of Egr-1 would decrease ICAM-1 levels.

In addition, it was known that "[p]rogressive interstitial fibrosis accompanied by loss of renal tubules and interstitial capillaries typifies all progressive renal diseases." Eddy "Molecular insights into renal interstitial fibrosis" *Journal of the American Society of Nephrology* 7:2495-2508 (1996) at page 2495, abstract. Fibronectin, PDGF, and collagen I are examples of fibrosis-related genes. *See id.* at pages 2496, Table 4, and 2502, right column; *see also* specification at page 9, lines 5-10. Thus, lowering the levels of these fibrosis-related gene would be expected to provide a therapeutic effect against renal disease. The specification shows that Candasartan decreases Egr-1, PDGF-B, fibronectin, and α1(I) collagen mRNA expression in Zucker fatty rats with increased urinary albumin excretion (Experimental Example 2). Experimental Example 1 also shows increased Egr-1 and fibronectin mRNA in Wistar fatty rats with diabetic nephropathy, and Experimental Example 5 shows increased Egr-1 protein and fibronectin mRNA

in HEK-293 cells transfected with a human Egr-1 expression plasmid, suggesting that decreases in these fibrosis-related genes would alleviate renal disease.

It was also known that "microalbuminuria[, a urinary albumin excretion rate of >30 mg/24 h and ≤300 mg/24 h] strongly predicts the development of nephropathy in patients with IDDS or NIDDM" and that "persistent albuminuria (>300 mg/24 h or 200μg/min) is the hallmark of diabetic nephropathy." Parving "Microalbuminuria in essential hypertension and diabetes mellitus" *Journal of Hypertension Suppl.* 14(2):S89-93 (1996) at page S91 and at page S89, respectively. According to Parving:

> [F]indings suggest that albuminuria can be used a so-called surrogate endpoint, that is, one that is correlated with and predicts a principal endpoint, for example, the rate of decline in glomerular filtration. A principal endpoint is defined as an endpoint is defined as an endpoint which is of benefit to the patient if improved, such as the rate of glomerular filtration.

Id. at page S89. Here, the Examples show increased urinary albumin excretion in Wistar fatty rats (Experimental Example 1) and in Zucker fatty rats (Experimental Example 2) compared to their lean rat controls. Accordingly, the fatty rats had renal disease, as indicated by the increased urinary albumin excretion. Treatment with Candesartan decreased the urinary albumin excretion and Erg-1, thus producing a therapeutic effect for a renal disease (Example 2).

It was also known that:

> [T]he administration of an antiserum capable of neutralizing TGF-β or of decorin, a natural TGF-β inhibitor, prevented the increased production of matrix proteins by glomerular cells and blocked ECM accumulation in a rat model of mesangial glomerulonephritis. It was also shown that in vivo transfection of the TGF-β gene into the rat kidney led to increased production of TGF-β_1 in glomeruli associated with a rapid development of glomerulosclerosis.

Rivarola et al. "Transforming growth factor beta activity in urine of patients with type 2 diabetes and diabetic nephropathy" *Braz J Med Biol Res* 32(12): 1525-1528 (1999) at page 1526. As noted above, the instant specification shows increased TGF-β levels and Egr-1 mRNA levels in animal models with renal disease, e.g., Wistar fatty rats (Experimental Example 1) and Zucker fatty rats (Experimental Example 2). Again, treatment with Candesartan decreased TGF-β and Egr-1 mRNA levels (Experimental Example 2). Thus, reduction in TGF-β mRNA levels indicated a therapeutic effect.

Thus, the specification combined with the knowledge of one skilled in the art does provide evidence that an agent that alters expression of Egr-1 produces a therapeutic effect in a disease model. Based on the above, practicing the method of instant claim 1 would not require undue experimentation. Applicants respectfully request withdrawal of the rejection.

II. Rejection of Claims 1, 5, and 6 under 35 U.S.C. § 103(a)

Claims 1, 5, and 6 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Rupprecht et al. (2000) Kidney Int. 57:70-82 ("Ruprecht") in view of McKay et al. (1998) *Anal. Biochem.* 256:28-34 ("McKay") and further in view of Raugi et al. (1987) *Am. J. Pathol.* 129:364-372 ("Raugi").

According to the Examiner, Rupprecht teaches five aspects of the instant claims. First, the Examiner alleges that Rupprecht teaches "a method comprising comparing expression of an Egr-1 protein in rat glomerular mesangial cells in the absence (i.e., serum only) and presence of a test compound." Action at page 15. Second, Rupprecht allegedly teaches "a method comprising comparing expression of a reporter gene under the transcriptional control of Egr-1 in the absence and presence of a test compound." *Id.* Third, the Examiner states that Rupprecht teaches "a method comprising comparing binding of Egr-1 to a polypeptide comprising the Egr-1 binding

site wherein the method uses the polynucleotide and an antibody against Egr-1 (i.e., supershifting)." *Id.* Fourth, the Examiner also states that Rupprecht teaches "that GSNO inhibits the expression and DNA binding activity of Egr-1 to DNA." *Id.* Fifth, the Examiner further states that Rupprecht "teaches the method using rat cells and therefore the polypeptide comprises the sequence of the rat Egr-1 ortholog." *Id.*

The Examiner acknowledges that the method of Rupprecht "does not teach the steps of immobilizing a polynucleotide on a solid support and contacting the solid phase with the protein and an antibody against the protein." Action at page 15.

The Examiner contends that McKay teaches "that ELISA assays for determining the DNA binding activity of DNA binding proteins involving immobilization of a target DNA to a solid support and contacting the solid support with the DNA binding protein and an antibody that binds to the DNA binding protein were known in the art." Action at page 16. The Examiner also contends that McKay teaches that "the ELISA assay described therein is an art recognized alternative to the EMSA assay." *Id.*

The Examiner urges that Raugi "demonstrate[s] that cultured human glomerular mesangial cells, which inherently express the Egr-1 polypeptide comprising SEQ ID NO:2 were available in the art and used in methods of characterizing mesangial cell function at the time the instant invention was made." Action at page 16.

The Examiner concluded that it "would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the method of Rupprecht '00 by substituting ELISA assay of McKay et al. for the EMSA assay used in the method of Rupprecht '00 and the human glomerular mesangial cells of Ragui et al. for the rat glomerular mesangial cells of

Rupprecht '00." Action at page 16. Relying on *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 (US 2007), the Examiner also stated that:

> [A]ll of the elements of the method and there [sic] uses were known to one of ordinary skill in the art at the time the invention was made and the skilled artisan could have substituted one known element for another element known in the art to obtain the predictable outcome of an assay for determining the effects of an agent on human Egr-1 expression and binding to DNA.

Id. at page 17.

Applicants respectfully traverse. The Supreme Court recently reaffirmed the framework set forth in *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966) for applying the statutory language of 35 U.S.C. § 103:

> Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. *Id., at 17-18, 86 S. Ct. 684, 15 L. Ed. 2d 545.*

KSR Int'l Co. v. Teleflex Inc., 127 S.Ct. 1 727, 1734 (2007), quoting *Graham*, 383 U.S. at 17-18. The Supreme Court further explained that "the factors continue to define the inquiry that controls." *Id.*

The *Graham* test reinforces Applicants' assertion that the claims are not obvious. Contrary to the Examiner's position, the cited documents do not provide or suggest all of the elements of instant claim 1. For example, the cited documents to not provide a "method of screening for a therapeutic substance for a renal disease." Nor do the cited documents provide any reason for "selecting the compound of step (f) that decreases the level of at least one selected

from urinary albumin excretion, TGF-β1, ICAM-1, tissue factor, PDGF-B, fibronectin, and α1 (I) collagen."

Rupprecht '00, the primary reference, does not discuss or suggest a method of screening comprising the elements of the instant claims. Instead, Rupprecht '00 assessed "whether GSNO[, S-nitrosoglutathione,] directly interfered with the DNA-binding activity or the transcriptional-activating capability of Egr-1," Rupprecht '00 at page 71, right column. While Rupprecht '00 does discuss a relationship between Egr-1 and mesangial proliferation, Rupprecht '00 does not teach or suggest a step of "selecting the compound of step (f) that decreases the level of at least one selected from urinary albumin excretion, TGF-β1, ICAM-1, tissue factor, PDGF-B, fibronectin, and α1 (I) collagen."

The secondary references do not compensate for the deficiencies of Rupprecht '00. McKay discusses using ELISA to measure binding of transcription factors to DNA, but does not discuss "a therapeutic substance for a renal disease" or "selecting the compound of step (f) that decreases the level of at least one selected from urinary albumin excretion, TGF-β1, ICAM-1, tissue factor, PDGF-B, fibronectin, and α1 (I) collagen."

Raugi, on the other hand, "examined cultured human glomerular mesangial cells for the ability to synthesize and secrete thrombospondin." Raugi at page 364, abstract. Like McKay, Raugi does not teach or suggest screening for "a therapeutic substance for a renal disease" or "selecting the compound of step (f) that decreases the level of at least one selected from urinary albumin excretion, TGF-β1, ICAM-1, tissue factor, PDGF-B, fibronectin, and α1 (I) collagen." Thus, the combination of Rupprecht, McKay, and Raugi does not provide all of the claim elements.

In addition, Rupprecht, McKay and Raugi do not teach or suggest "measuring production of the protein or salt thereof and the binding activity of the protein or salt thereof to the polynucleotide" in a single assay. Specifically, Rupprecht discusses measuring Egr-1 levels using Western blot assays and binding activities using EMSA. *See* Figure 5 at page 76 and Figure 7 at page 78, respectively. McKay discusses measuring NF-IL6 activity using ELISA, e.g., at page 29, but does not discuss "measuring production" of any transcription factors. Raugi, on the other hand, discusses detecting thrombospondin production using immunostaining and metabolic labeling techniques. Thus, the cited references, alone or in combination, do not describe "measuring production of the protein or salt thereof and the binding activity of the protein or salt thereof to the polynucleotide" in a single assay.

Accordingly, the combination of Rupprecht '00, McKay, and Raugi do not render the instant claims obvious. Applicants respectfully request withdrawal of the rejection.

III. Conclusion

Applicants respectfully request that this Amendment under 37 C.F.R. § 1.116 be entered by the Examiner, placing claims 1 and 6 in condition for allowance.

It is respectfully submitted that the entry of the Amendment would allow the Applicants to reply to the final rejections and place the application in condition for allowance.

Finally, Applicants submit that the entry of the amendment would place the application in better form for appeal, should the Examiner dispute the patentability of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: October 29, 2008

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Microalbuminuria in essential hypertension and diabetes mellitus

Hans-Henrik Parving

Definition Microalbuminuria is defined as abnormally elevated urinary albumin excretion below the level of clinical albuminuria (albustix). This represents a urinary albumin excretion rate of 20–200 μg/min, equal to 30–300 mg/24 h. Urinary albumin excretion can vary as much as 40% with natural fluctuations, and so several tests should be done. Inexpensive radioimmunoassay, enzyme-linked immunosorbent assays or immunoturbidimetric assays are now routine in many clinical laboratories.

Prevalence The prevalence of microalbuminuria in essential hypertension and diabetes is about the same: 25% (range 14–31) and 20% (9–27), respectively.

Mechanisms Increased transglomerular passage is the major mechanism of microalbuminuria in both the above-mentioned conditions; increased hydraulic glomerular capillary pressure and glomerular lesions probably both contribute. Microalbuminuria is highly predictive of the development of diabetic nephropathy but the predictive power in relation to hypertensive nephropathy remains to be established. However, in both conditions microalbuminuria is associated with an increased risk of retinopathy, left ventricular hypertrophy, fatal and non-fatal cardiovascular disease and all-cause mortality. The following mechanisms have been suggested as a link between microalbuminuria and these findings: endothelial dysfunction, insulin resistance, hyperinsulinemia, dyslipoproteinemia and a procoagulant state.

Effect of antihypertensive treatment Blood pressure lowering reduces microalbuminuria in essential hypertension and in diabetes mellitus. Long-term studies in diabetes suggest that angiotensin converting enzyme inhibitors postpone, and may even prevent, progression to overt clinical nephropathy in normotensive diabetic patients with persistent microalbuminuria. So far, there have been no long-term comparative trials on the beneficial effects of different antihypertensive drugs in hypertensive patients with microalbuminuria.

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Keywords: diabetes mellitus, microalbuminuria, diabetic nephropathy, antihypertensive treatment, essential hypertension, hypertensive nephropathy

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Introduction

Persistent albuminuria (>300 mg/24 h or 200 μg/min) is the hallmark of diabetic nephropathy, which can be diagnosed clinically if the following additional criteria are fulfilled: presence of diabetic retinopathy and no clinical or laboratory evidence of kidney or urinary tract disease other than diabetic glomerulosclerosis [1]. This clinical definition of diabetic nephropathy is valid in insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetic patients. Albuminuric NIDDM patients without retinopathy require further evaluation by kidney biopsy, since the ratio of the risk of non-diabetic to diabetic glomerulopathy is approximately 50 : 50 [2]. The clinical syndrome termed diabetic nephropathy is characterized by persistent albuminuria, early blood pressure elevation, a relentless decline in the glomerular filtration rate and a high risk of cardiovascular morbidity and mortality. Hypertensive nephropathy is generally accepted as having the following clinical features: minimal albuminuria (<1000 mg/24 h), other end-organ evidence of long-standing hypertension such as left ventricular hypertrophy, a strong family history of hypertension and, most important, a normal renal function at the time of the onset of essential hypertension, thus excluding renal disease in the etiology of the hypertension [3]. Consequently, hypertensive nephropathy is an exclusion diagnosis. Diabetes mellitus and hypertension are the major causes of end-stage renal failure in the United States and Europe. Proteinuria is a classical sign of glomerulopathy but, in addition, proteinuria accelerates the progression of hypertensive and diabetic glomerulopathy [4,5].

Recent evidence indicates that a reduction in albuminuria during antihypertensive treatment predicts an attenuated rate of decline in the glomerular filtration rate in diabetic and non-diabetic glomerulopathies [6,7]. These findings suggest a clinical application in monitoring the efficacy of antihypertensive treatment in glomerulopathy. The findings suggest that albuminuria can be used as a so-called surrogate endpoint, that is, one that is correlated with and predicts a principal endpoint, for example the rate of decline in glomerular filtration. A principal endpoint is defined as an endpoint which is of benefit to the patient if improved, such as the rate of decline in glomerular filtration. Several longitudinal studies have shown that microalbuminuria, defined as a urinary albumin excretion rate of >30 mg/24 h and ≤300 mg/24 h, strongly predicts development of diabetic nephropathy in IDDM and NIDDM patients [1]. Similar data are not yet available for essential hypertension.

The aim of the present review is to compare epidemiology, prognosis, pathophysiology, and antihypertensive treatment

Table 1 Prevalence, incidence and cumulative incidence of microalbuminuria and nephropathy in essential hypertension and diabetes mellitus

	References	Essential hypertension	IDDM	NIDDM
Prevalence of microalbuminuria (%)	[1,8–15]	25 (14–31)	13 (9–20)	25 (13–29)
Prevalence of macroalbuminuria (%)	[14–17]	14 (4–18)	15 (8–22)	14 (5–48)
Incidence of macroalbuminuria (%/year)	[18]	2	1.2 (0–3)	1.5 (1–2)
Cumulative incidence of macroalbuminuria (%/25 years)	[19]	42	31 (28–34)	28 (25–31)

Medians and ranges (parentheses) are given. IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus. In essential hypertension the cumulative incidence of macroalbuminuria represents a lifelong incidence [19].

of microalbuminuric patients with essential hypertension and diabetes mellitus.

Detection and monitoring of microalbuminuria

Microalbuminuria is defined as a urinary albumin excretion of 30–300 mg/24 h or 20–200 μg/min. The term persistent microalbuminuria is commonly used if the urinary albumin excretion is within this range in two out of three consecutive timed urine collections. Several procedures for urine collections have been suggested: 24-h urine collection, overnight collection, short-term collection in the clinic and measuring the urinary albumin : creatinine ratio in random or early morning urine. A urinary albumin : creatinine ratio of 30–300 mg/g or 2.5–25 mg/mmol is suggestive of microalbuminuria. The ratio can be used for screening and also for monitoring treatment, but confirmatory tests are best performed by the use of timed urine collections. Urinary albumin excretion can vary by as much as 40% with natural fluctuations. Inexpensive radioimmunoassays, enzyme-linked immunosorbent assays or immunoturbidimetric assays are now routine in many laboratories.

There are several confounding factors in a diagnosis of microalbuminuria, including massive obesity, heavy exercise, urinary tract infection, various acute and chronic illnesses, cardiac failure and the use of various drugs [for example non-steroidal anti-inflammatory drugs (NSAID) reduce albuminuria by reducing levels of vasodilating prostaglandins].

Epidemiology of microalbuminuria

The prevalence of microalbuminuria varies between 14 and 31%, with a mean value of 25% in essential hypertension [8–15]. The prevalence of microalbuminuria in NIDDM patients is within the same range, but slightly lower in IDDM patients [1], as demonstrated in Table 1.

The prevalence of macroalbuminuria (>300 mg/24 h) is nearly identical in essential hypertension [14,16,17] and diabetes [1], with a median value of 15% but the range is rather wide, probably reflecting referral bias. There is scant information on the incidence of macroalbuminuria (2%/year) in essential hypertension [18], but several studies in diabetes have demonstrated a yearly incidence ranging between 1 and 2% [1]. The cumulative incidence of macroalbuminuria after a 25-year duration of diabetes is 30%, which is comparable with the findings of Perera [19] of 42% in the natural history of essential hypertension.

Table 2 Microalbuminuria as predictor of microangiopathy, macroangiopathy, nephropathy and mortality in essential hypertension and diabetes mellitus

		Microalbuminuria	
	References	Essential hypertension	Diabetes mellitus
Retinopathy	[1,14]	+	+
LVH	[1,12,23]	+	+
Non-fatal CVD	[1,20–22]	+	+
All-cause mortality	[1,20,21]	+	+
Nephropathy	[1]	?	+
Peripheral/autonomic neuropathy	[1]	?	+

LVH, left ventricular hypertrophy; CVD, cardiovascular disease.

Table 3 Factors known to be associated with microalbuminuria in essential hypertension and diabetes mellitus

		Microalbuminuria	
	References	Essential hypertension	Diabetes mellitus
Endothelial dysfunction	[1,24]	+	+
Procoagulant state	[1,24]	+	+
Increased platelet aggregability	[1]	?	+
Insulin resistance	[1,20,25]	+	+
Hyperinsulinemia	[1,20,25]	+	+
Dyslipoproteinemia	[1,14,16]	+	+
Blood pressure	[1]		+
Short stature	[26,27]	+	+

Prognosis in microalbuminuria

Several retrospective and prospective studies have demonstrated that microalbuminuria independently predicts cardiovascular morbidity and all-cause mortality in essential hypertension [14,20–22] and diabetes mellitus (Table 2) [1,12,14,20–23]. Patients with microalbuminuria are also characterized by an increased prevalence of left ventricular hypertrophy and retinal microvascular lesions [12,23]. The links between microalbuminuria and death from cardiovascular disease are poorly understood. Several explanations have been proposed. One possible explanation is that since microalbuminuria is a marker of widespread endothelial dysfunction this might promote increased penetration of atherogenic lipoprotein particles in the arterial wall which, in turn, is a marker of established cardiovascular disease. Microalbuminuria is associated with an excess of known and potential cardiovascular risk factors (Table 3) [1,14,16,20,24–27]. Dyslipoproteinemia, insulin resistance and hyperinsulinemia have also been demonstrated in microalbuminuric patients with essential hypertension and diabetes [20,25]. Autonomic

neuropathy, which is also associated with microalbuminuria, predicts death (often sudden) from cardiovascular disease, at least in diabetic patients. Left ventricular hypertrophy is more frequent in microalbuminuric patients with essential hypertension [12,23] and diabetes mellitus [1] (Table 2), and left ventricular hypertrophy predisposes the patient to ischemic heart disease, ventricular arrythmia, heart failure and sudden death.

Microalbuminuria predicts nephropathy

Several longitudinal studies have shown that microalbuminuria strongly predicts the development of nephropathy in patients with IDDM or NIDDM [1]. IDDM patients with microalbuminuria have a median rate of risk of 21 for developing nephropathy while the risk ratio ranges between 4 and 21 (medium 9) in microalbuminuric NIDDM patients. At present, we have no retrospective or prospective data that indicate the predictive power of microalbuminuria in essential hypertension.

Renal biopsy studies have demonstrated varying degrees of glomerular lesions in microalbuminuric IDDM and NIDDM patients. Unfortunately, we have no information on this important association between an abnormally elevated urinary albumin excretion rate and glomerulopathy/vasculopathy in essential hypertension.

Pathophysiology and antihypertensive treatment of microalbuminuria

The rate of excretion of albumin in the urine is determined by the amount filtered across the glomerular capillary barrier minus the amount reabsorbed by the tubular cells. A normal urinary β_2-microglobulin excretion rate in hypertensive [8] and diabetic patients [1] suffering from microalbuminuria suggests that the rate is caused by increased glomerular leakage rather than a reduced tubular reabsorption of protein. The transglomerular passage of macromolecules is governed by the size- and charge-selective properties of the glomerular capillary membrane and by hemodynamic forces operating across the capillary wall. Alterations in glomerular pressure and flow influence both the diffusive and convecting driving forces for transglomerular passage of proteins. Glomerular hydraulic pressure cannot be measured in man, but the filtration fraction is presumed to reflect glomerular pressure. Microalbuminuric patients with essential hypertension and diabetes both have an elevated filtration fraction and a close correlation between the filtration fraction and urinary albumin excretion has also been demonstrated. The positive correlation between arterial blood pressure and the urinary albumin excretion rate suggests that increased glomerular filtration of proteins is involved [8,28,29]. Furthermore, the demonstration that microalbuminuria falls promptly with an acute reduction in arterial blood pressure indicates that reversible hemodynamic factors are important in the pathogenesis of microalbuminuria, both in hypertensive and diabetic subjects. However, normal urinary albumin excretion is not induced by lowering blood pressure to normal values, suggesting that irreversible so-called target-organ damage (glomerulopathy) may also contribute [28]. Animal studies have clearly demonstrated that glomerular capillary hydraulic pressure is increased in streptozotocin-diabetic rats and hypertensive rats [30–32]. In addition, changes in the charge-selective properties have been demonstrated in diabetic patients with microalbuminuria [1].

Until recently, the adverse impact of systemic hypertension on renal structure and function was thought to be mediated through vasoconstriction and arteriolar nephrosclerosis. However, evidence from rat models shows that systemic hypertension is transmitted to the single glomerulus in such a way as to lead to hyperperfusion and increased glomerular capillary pressure [30–33]. The intraglomerular hemodynamics are probably similar to those found in other models of disease progression where systemic blood pressure is minimally or only slightly increased, but glomerular perfusion and glomerular pressure are increased [32]. The reduction in glomerular injury associated with antihypertensive therapy in many of these experimental models supports this view [31,32].

Several trials have been conducted in microalbuminuric patients with essential hypertension. Nearly all the trials conducted are randomized, double-blind, crossover trials of short duration (weeks) comparing different antihypertensive drugs [28,34–37]. There is scant information from randomized, double-blind, parallel studies comparing different blood pressure lowering agents [38,39]. The results from all these trials suggest that a reduction in blood pressure reduces but does not normalize urinary albumin excretion. Some but not all trials have reported that angiotensin converting enzyme (ACE) inhibition has a more beneficial effect than other classes of antihypertensive drugs [38,39]. However, there are no data from long-term (>1 year), randomized, double-blind, parallel studies comparing different antihypertensive agents in microalbuminuric patients with essential hypertension. In contrast, numerous trials have been conducted in diabetes mellitus, particularly in IDDM patients, demonstrating that ACE inhibitors are superior to other antihypertensive drug classes in reducing the urinary albumin excretion rate, both in the micro- and in the macroalbuminuric range [1,40]. This suggests a so-called renoprotective effect, defined as a beneficial effect on renal function and structure above and beyond that obtained by the blood pressure fall itself.

In the past decade, several randomized, parallel studies comparing ACE inhibitors with placebo have been conducted in normotensive microalbuminuric IDDM patients [41–43]. All the long-term studies (>1 year) have shown that ACE inhibitors have a beneficial effect in postponing the development of overt diabetic nephropathy. Furthermore, a recent 8-year prospective study has shown that the beneficial effect of ACE inhibition is long-lasting [44]. The impact of ACE inhibition

in normotensive microalbuminuric NIDDM patients has also been evaluated. Ravid *et al.* [45] conducted a double-blind, randomized study in 94 normotensive microalbuminuric NIDDM patients given enalapril or placebo for 5 years. In the actively treated group, kidney function remained stable and diabetic nephropathy developed in only 12% of the patients. However, in the group given placebo treatment, 42% developed nephropathy and kidney function declined by 13%.

A cost–benefit analysis of screening and antihypertensive treatment for early renal disease as indicated by microalbuminuria in diabetes showed that screening and intervention program are likely to have life-saving effects and lead to considerable economic savings [46]. In the past year six different sets of recommendations on the prevention of diabetic nephropathy, with special reference to microalbuminuria, have been published, as reviewed by Mogensen *et al.* [47].

An agreed, unifying, global strategy has been recommended previously [8]. This included annual screening for microalbuminuria in all adult diabetic patients below the age of 70 years, abnormal screening values to be confirmed by timed urine collections taking into account the high day-to-day variability and various confounding variables. If persistent microalbuminuria is confirmed, treatment with ACE inhibition should be initiated in an attempt to stabilize or even reduce microalbuminuria, preserve kidney function and thus postpone diabetic nephropathy and end-stage renal disease. This paper [8] introduced the concept of antihypertensive renalprotective treatment, even when blood pressure is not at a conventional hypertensive level. The program outlined is very cost-effective, mainly because end-stage renal treatment is so expensive.

The available data in essential hypertension suggest that screening and monitoring of microalbuminuria is worthwhile in order to identify a high-risk subpopulation and to monitor antihypertensive treatment for its effect on target-organ damage. However, so far, no information is available on antihypertensive treatment in these patients in relation to the development of end-stage renal failure.

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Discussion

J.L. Rodicio (Madrid, Spain): For screening purposes, the microalbuminuria-creatinine relaionship in urine collected first thing in the morning is a good parameter. But what about for quantification? Do you recomend 24 h or overnight urine collection?

H.H. Parving (Gentofte, Denmark): A timed collection is necessary for quantification, either 24 h or overnight. Overnight collection is standardized, simple to perform and preferred by many people because there is no need to carry a bottle for urine collection.

J.L. Rodicio (Madrid, Spain): Is there any difference, during overnight collection, between dippers and non-dippers?

H.H. Parving (Gentofte, Denmark): We have studied dippers and non-dippers in diabetes in relation to the overnight excretion rate and we did not find any differences.

K.H. Rahn (Munster, Germany): Glomerular capillary pressure was mentioned as a main determinant of albumin excretion via the kidney. Is it not rather the transmembrane pressure gradient at the level of the glomerular basement membrane? This parameter could also be altered by changes in the intratubular pressure.

H.H. Parving (Gentofte, Denmark): Quite right.

L. Hansson (Uppsala, Sweden): The excretion of microalbuminuria appears to be almost directly related to the level of pressure, and the exercise data supported this. Is there a difference between day and night excretion, as might be expected? If that is the case, might this be simply a marker of the blood pressure level but one which is more difficult to measure and more costly to measure than direct blood pressure measurements?

H.H. Parving (Gentofte, Denmark): There are differences between patients. In the normoalbuminuric state, in the microalbuminuric state and in the state with overt microalbuminuria there are rather big fluctuations from day-time to night-time. Protein excretion at night is approximately 40% lower than during the day. We have been doing 24-h measurements in order to correlate the excretion of albumin with 24-h blood pressure. We have found a close correlation between daytime blood pressure and daytime albuminuria, and there is a close correlation between night-time blood pressure and night-time albuminuria. But the reduction from day to night could not explain the change in albuminuria from day to night, and that was rather disturbing.

We are now studying fractional albumin clearance, by measuring albumin and also the glomerular filtration rate during the day and at night; glomerular filtration falls by 20–25% at night. Our earlier result may be an artefact, in that the albuminuria is not falling and that much is a result of a fall in the filtered load.

L. Hansson (Uppsala, Sweden): You showed that the reduction in microalbuminuria was greatest in the II (insertion/insertion) genotype group which also showed the greatest drop in blood pressure. It would make sense to express the reduction in proteinuria in relation to the fall in blood pressure per mmHg, and I think there would be no difference.

H.H. Parving (Gentofte, Denmark): I think so. What we showed in this study, which was finished just a couple of days ago, is that there was a clear correlation between the drop in blood pressure and the drop in albuminuria during the trial. Of

course, a major part of the large fall in albuminuria in the II genotype group, which also had the greatest fall in blood pressure, was probably due to hemodynamic phenomena. However, we also found that blood pressure, albuminuria itself and genotype all independently predicted the drop in albuminuria. There is other evidence in non-diabetic kidney disease from Japan; Yoshida *et al* [48] also demonstrated a relationship between the genotype and the antiproteinuric effect. This study was not complicated by differences in blood pressure. The most important finding is that genotype seems to be a very important risk marker in relation to the loss of kidney function in both diabetic and non-diabetic kidney disease. The DD (deletion/deletion) genotypes lose twice as much as the IIs and this is a hard end-point. This finding has been described in recent papers [49,50].

J. Ménard (Paris, France): We are conducting a study in France on this subject, the Diabetes, Hypertension and Cardiac disease (DIAB HYCAR) study, chaired by Michel Marre. We are trying to determine whether ACE inhibitors, compared with placebo, can protect patients with NIDDM and microproteinuria from cardiovascular events. When we set up this study, some physicians said that because there are published recommendations concerning the beneficial effect of ACE inhibitors, all diabetic patients with microproteinuria should be treated with an ACE inhibitor and our study is probably unethical.

H.H. Parving (Gentofte, Denmark): The background to the *Lancet* paper [47] is that there had been six different conferences where different recommendations were made and finally there was a decision to make one global recommendation.

G. Mancia (Milan, Italy): Differences between day and night values for microalbuminuria may be explained by the fact that pressure transmission to the glomeruli is different at night compared to the daytime because of differences in vasomotor tone in the afferent arteriole.

It seems that in diabetics a reduction in microalbuminuria gives renal protection, so we have a case in which a surrogate end-point has proved to be a real end-point. Does this hold only for ACE inhibitors or does it hold for any drug that reduces microalbuminuria?

H.H. Parving (Gentofte, Denmark): I agree that changes in the tone of the arterioles could very well explain some of the differences in albumin excretion rates.

In answer to the second question, ACE inhibitors have no monopoly. If other kinds of compound can also reduce microalbuminuria to the same extent, then the renoprotective effect should be the same.

G. Mancia (Milan, Italy): A reduction in microalbuminuria is nephroprotective, and a reduction in blood pressure has cardiovascular protection. Therefore a reduction in blood pressure is only an intermediate end-point, also. Yet treatment of high blood pressure is fully accepted.

H.H. Parving (Gentofte, Denmark): This is one of our concerns. The United States Food and Drug Administration (FDA) is discussing the question of treatment for microalbuminuria, and in some countries microalbuminuria has been accepted as a valid endpoint. From the state of microalbuminuria until a principal endpoint is reached there is a wait of at least 10–15 years or maybe even longer. Nevertheless, the number of patients with diabetes and hypertension who reach end-stage renal failure is extraordinarily high. At that point it is too late to treat them.

P.A. van Zwieten (Amsterdam, the Netherlands): What is the mechanism of the suppression of microalbuminuria by NSAID?

H.H. Parving (Gentofte, Denmark): It is a reduction in vasoactive prostaglandins. The vasoactive prostaglandin dilates the afferent arteriole and the pressure falls in the glomerulus. Then there is constriction and an immediate reduction in albuminuria, approximately 50%.

P.A. van Zwieten (Amsterdam, the Netherlands): Does aspirin have the same effect?

H.H. Parving (Gentofte, Denmark): Sure.

J.L. Rodicio (Madrid, Spain): It depends on the dosage of aspirin.

H.H. Parving (Gentofte, Denmark): There is probably some kind of a dose relationship, but I do not know the exact dose. One gram of aspirin three times a day is a large dose.

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Transforming growth factor beta activity in urine of patients with type 2 diabetes and diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is characterized structurally by progressive mesangial deposition of extracellular matrix (ECM). Transforming growth factor-ß (TGF-ß) is considered to be one of the major cytokines involved in the regulation of ECM synthesis and degradation. Several studies suggest that an increase in urinary TGF-ß levels may reflect an enhanced production of this polypeptide by the kidney cells. We evaluated TGF-ß in occasional urine samples from 14 normal individuals and 23 patients with type 2 diabetes (13 with persistent proteinuria >500 mg/24 h, DN, 6 with microalbuminuria, DMMA, and 4 with normal urinary albumin excretion, DMN) by enzyme immunoassay. An increase in the rate of urinary TGF-ß excretion (pg/mg $U_{Creat.}$) was observed in patients with DN (296.07 ± 330.77) ($P<0.001$) compared to normal individuals (17.04 ± 18.56) (Kruskal-Wallis nonparametric analysis of variance); however, this increase was not observed in patients with DMMA (25.13 ± 11.30) or in DMN (18.16 ± 11.82). There was a positive correlation between the rate of urinary TGF-ß excretion and proteinuria ($r = 0.70$, $\alpha = 0.05$) (Pearson's analysis), one of the parameters of disease progression.

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Key words
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- Proteinuria
- Diabetic nephropathy
- Extracellular matrix

Diabetic nephropathy (DN) is characterized structurally by renal hypertrophy and by progressive mesangial deposition of extracellular matrix (ECM) characterizing glomerulosclerosis (1), which is associated with progressive glomerular capillary occlusion, albuminuria and a progressive fall in glomerular filtration rate (GFR). These alterations are probably of multifactorial etiology, and hemodynamic, metabolic, or genetic factors have been proposed to explain them (2).

Several lines of experimental and clinical evidence suggest that transforming growth factor-ß (TGF-ß) may play a causative role in the development of glomerulosclerosis and interstitial fibrosis observed in the course of many renal diseases (3-6). TGF-ß has been considered as a major cytokine involved in the regulation of ECM synthesis and degradation (7). This polypeptide can stimulate the synthesis of ECM components, including collagen, fibronectin and proteoglycans (7,8) and block matrix degradation (7), thus promoting ECM accumulation. Overproduction of TGF-ß has been observed in many exper-

imental and human kidney diseases (5,6,9-11). Border et al. (3,5) reported that the administration of an antiserum capable of neutralizing TGF-ß or of decorin, a natural TGF-ß inhibitor, prevented the increased production of matrix proteins by glomerular cells and blocked ECM accumulation in a rat model of mesangial glomerulonephritis. It was also shown that *in vivo* transfection of the TGF-ß gene into the rat kidney led to increased production of TGF-$ß_1$ in glomeruli associated with a rapid development of glomerulosclerosis (6).

The results of some clinical and experimental studies have suggested that the measurement of urinary TGF-ß content could be a useful noninvasive procedure for the evaluation of the renal production of this polypeptide (12,13). In a previous study we observed an increase in urinary TGF-ß activity in patients with glomerulonephritis (13). In the present study we evaluated TGF-ß activity in urine from patients with type 2 diabetes and its correlations with albuminuria and plasma creatinine levels.

Patients were selected from those attending our outpatient clinic or admitted to Hospital das Clínicas de Ribeirão Preto. Spot urine samples were collected from 23 patients with type 2 diabetes (13 with persistent proteinuria >500 mg/24 h, DN, 6 with microalbuminuria, urinary albumin excretion rate >20 and <200 µg/min, DMMA, and 4 with normal urinary albumin excretion, DMN) and from 14 normal individuals. The age range of the 23 patients was 19-57 years and the age range of the controls was 22-51 years. The range of creatinine levels of our patients was 0.6-3.0 mg/dl and the range of proteinuria 0.06-4.5 g/24 h. Plasma glucose levels assessed by the glycosylated hemoglobin test were well controlled in all patients. The patients had not been taking angiotensin II (AII) converting enzyme inhibitor or AII antagonist drugs for at least 15 days before urine collection. After TGF-ß determination, these medications were introduced for some of these patients. All control subjects had normal albuminuria, plasma creatinine levels and arterial pressure. The research was approved by the Ethics Committee of Hospital das Clínicas de Ribeirão Preto and informed consent was obtained from all individuals.

The urine samples were treated with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA) and aliquots were taken for urine creatinine determination and centrifuged at 2,000 rpm in a clinical centrifuge for 5 min at 4°C. To measure total (latent + active) TGF-ß activity, HCl was added to the sample until pH 2.5 to 2.0 was obtained. After 30 min at room temperature, the pH was readjusted to 7.4 with NaOH. Quantification of TGF-ß in these samples was performed by enzyme-linked immunosorbent assay (ELISA) using kits from Promega Corp. (Madison, WI, USA) (14).

Urinary TGF-ß excretion is reported as urinary TGF-ß excretion/mg urinary creatinine to correct the variation in urine concentration. To construct a standard curve, known amounts of human recombinant TGF-$ß_1$ (Promega Corp., Madison, WI, USA) were added to each well. The response was linear and presented a positive correlation between the amount of this polypeptide and optic density ($r = 0.998$).

Albumin was measured by electroimmunoassay in urine samples collected during a 24-h period (15) using an antibody against human albumin. The investigation was performed 3 times in each patient. Urinary creatinine was measured by the method of Jaffé (16).

The nonparametric analysis of variance proposed by Kruskal-Wallis was used to compare the various groups, with the level of significance set at 0.05. The Pearson correlation coefficient was used to examine any significant correlation between the rate of urinary TGF-ß excretion and plasma creatinine levels and albumin in urine. Data are

expressed as mean ± SD.

A significant increase in the rate of urinary TGF-ß excretion (pg/mg $U_{Creat.}$) was observed in patients with type 2 diabetes with persistent proteinuria, DN (296.07 ± 330.77) ($P<0.001$) compared to normal individuals (17.04 ± 18.5); however, this increase was not observed in patients with microalbuminuria, DMMA (25.13 ± 11.30) or with normal albumin excretion, DMN (18.16 ± 11.82) (Figure 1).

A significant positive correlation was found between the amount of TGF-ß in urine and proteinuria in patients with type 2 diabetes (Figure 2) ($r = 0.70$, $\alpha = 0.05$). However, we did not find any correlation between the rate of urinary TGF-ß excretion and plasma creatinine levels.

The present results showed an increase in the rate of urinary TGF-ß excretion in patients with type 2 diabetes with persistent proteinuria (>500 mg/24 h). There was a wide variation in the rate of urinary TGF-ß excretion in these patients. However, these patients presented different stages and activity of renal disease.

Several studies have shown that TGF-ß may play a major role in glomerular disease, mediating the inflammatory response through glomerulosclerosis (4,5,9-11). Studies of *in situ* or *in vitro* hybridization and with immunohistochemical methods have demonstrated that the renal content of TGF-ß mRNA and TGF-ß protein was higher in many cases of experimental and clinical glomerulonephritis (4,5,9-11). The consequences of the increase in TGF-ß production are multiple since this protein is known to have important effects on the synthesis of several matrix components and can block matrix degradation, thus promoting ECM accumulation (7,8).

The pathogenetic mechanism(s) leading to increased renal TGF-ß production in diabetic patients are incompletely understood. Possibilities include glomerular hypertension and hypertrophy (1,2,17), an increase in intrarenal renin content (18) and higher levels of glucose in plasma (19). It has been reported that stretching of rat mesangial cells by mechanical strain can provoke increased ECM and TGF-ß production (17). It has been recently demonstrated that AII (18) and glucose (19) can stimulate mesangial secretion of TGF-ß. Some studies using mesangial cells in culture have shown that high-glucose media can stimulate mesangial secretion of TGF-ß, type IV collagen and other ECM components. It was also observed that AII stimulates rat glomerular mesangial cell production of type I collagen, fibronectin and biglycan through an induction of TGF-ß expression. Taken together, these data suggest that diabetic nephropathy may result from an interplay of mechanical, metabolic and humoral factors.

The present data show that the amount of TGF-ß in urine was significantly correlated

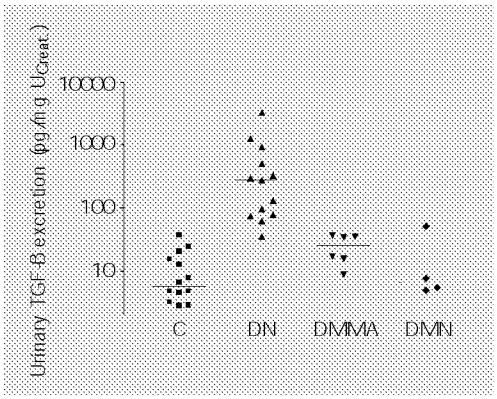

Figure 1 - Rate of urinary TGF-ß excretion for normal individuals (C, N = 14) and patients with type 2 diabetes with sustained proteinuria (DN, N = 13), with microalbuminuria (DMMA, N = 6) or with normal urinary albumin excretion (DMN, N = 4). TGF-ß in urine samples was measured by ELISA using kits from Promega. P = 0.001 for patients with type 2 diabetes (DN) compared to normal individuals and to DMMA and DMN (Kruskal-Wallis test). The median is shown as a horizontal line.

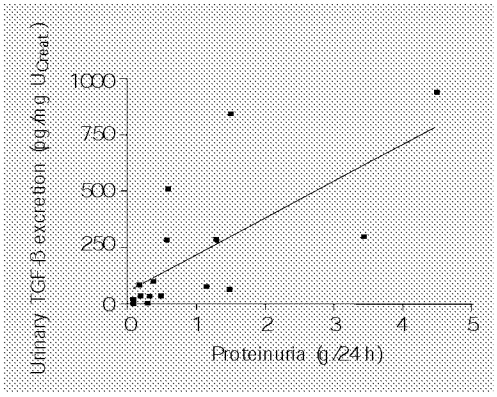

Figure 2 - Correlation between rate of urinary TGF-ß excretion and proteinuria observed in patients with type 2 diabetes. The correlation coefficient was r = 0.70 ($\alpha<0.05$; Pearson's analysis).

with proteinuria. There are some observations suggesting that urinary TGF-ß derives from renal biosynthesis and not from ultrafiltration or secretion (12,13,20). The amount of total TGF-ß (latent + active) is negligible in plasma and the amount of active TGF-ß is undetectable (20). Noh et al. (12) observed that urinary TGF-ß activity is related to the extent of scarring in crescentic nephritis in rabbits. In recent studies we observed a significant positive correlation between the rate of urinary TGF-ß excretion and the incidence of glomerulosclerosis in patients with glomerulonephritis (13). These data suggest that the increase of urinary TGF-ß activity observed in these patients may reflect enhanced production of this polypeptide by kidney cells or an increase in its renal content. Therefore, the measurement of the urinary activity of this polypeptide may be an early indicator for the evaluation of kidney disease progression.

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Adhesion molecules in renal disease

Adhesion molecules are a heterogeneous class of ligands/receptors that mediate cell adhesion, either to other cells or to the extracellular matrix. Cell adhesion is of fundamental importance to an impressive number of physiological and pathological processes, including the differentiation of cells and their organization in tissues [1], the intercommunication and activation of immune cells [2], the recirculation and migration of white blood cells [3], the growth and metastatic diffusion of tumoral cells [4]. On the basis of molecular, structural and functional differences, adhesion molecules have been separated into three main groups: the integrins, the selectins and a group that belongs to the immunoglobulin superfamily. In addition to these classic families of adhesion molecules, a recently described family of chemoattractive cytokines, termed chemokines, behave as adhesion molecules after having been released at a site of inflammation. These ligands, in fact, bind to specific receptors in the endothelium or extracellular matrix [5] and here regulate immune cell migration by haptotaxis, a process driven by the gradient of adhesive ligands affixed to the surface of cells or to the extracellular matrix [6].

This article first summarizes the features that distinguish the families of adhesion molecules and gives a concise description of their most relevant members. Then, the expression of adhesion molecules in renal cells in culture and in normal renal tissue, and the pathophysiological role of adhesion molecules in renal disease, with an emphasis on nephritis, transplant rejection and the effects of hemodialysis on leukocytes will be reviewed.

Families of adhesion molecules

Integrin family

The name integrins was originally coined to signify the role of these proteins in integrating the cytoskeleton with the extracellular matrix. Actually, integrins are very versatile and mediate both cell-to-matrix and cell-to-cell adhesion. All integrins are membrane glycoproteins consisting of two subunits, a larger alpha chain and a smaller beta chain [4]. A classification in subfamilies is based on the observation that some members have the same beta chain but different alpha chain. Thus, we distinguish $beta_1$ integrins (also called VLA, very late antigens, because some of them appear on lymphocytes 2 to 4 weeks after antigen stimulation) [7], $beta_2$ integrins (called leukocyte integrins because of their exclusive expression on leukocytes) [2] and $beta_3$ integrins or cytoadhesins [8]. In the last few years, however, new beta chains have been discovered, and it has been found that some alpha subunits are linked to more than one beta subunit, making the original classification insufficient [9].

All integrins conserve a strong sequence homology and structural and functional similarity. Thus, the ligand binding site is formed by short sequences on both chains [1]; the alpha chains have three or four divalent cation-binding motifs [2], and the intracytoplasmic domain interacts with the cytoskeleton through an association with talin and, perhaps, other cytoplasmic proteins. Many, but not all integrins recognize the specific amino-acid sequence RGD (Arg-Gly-Asp) and synthetic peptides with this sequence can block integrin-ligand interaction [2, 9].

The $beta_1$ integrins are formed by the combination of the $beta_1$ chain with different alpha chains and are numbered VLA-1 to VLA-6 according to the alpha chain number (α_1 to α_6). They are expressed on several cell types (leukocytes, platelets, fibroblasts, epithelial cells and endothelial cells) and typically bind to extracellular matrix proteins, mainly laminin, collagen and fibronectin. Thus VLAs promote attachment of endothelial and epithelial cells to substrata [9], adhesion of platelets to exposed subendothelial matrix, tissue repair, and localization of leukocytes in inflamed tissue [4]. Interesting and unusual is the behavior of VLA-4, an integrin expressed on lymphocytes, monocytes, basophils and eosinophils, that functions as a receptor for both fibronectin and VCAM-1, a ligand of the immunoglobulin superfamily expressed on endothelial cells. VLA-4 therefore plays a role in leukocyte-endothelial interactions.

The $beta_2$ integrins consist of three members that are exclusively expressed on leukocytes and are called LFA-1, Mac-1 and p150,95, respectively. LFA-1 is found on virtually all immunocytes, while Mac-1 and p150,95 are predominantly expressed on monocytes and granulocytes. These integrins are constitutively expressed in a nonactive conformation. Cell activation is followed by conformational change of the surface molecule and an increase in the avidity of the receptor, as well as by rapid mobilization from an intracellular pool and increased surface expression [10]. All three $beta_2$ integrins mediate adherence of leukocytes to endothelium, a fundamental step in leukocyte migration. LFA-1 is the best studied and, perhaps, the most important; its endothelial ligands are two proteins of the immunoglobulin superfamily: ICAM-1 and ICAM-2. In addition to endothelial cells, ICAM-1 is induced by activation in several other cell types, including lymphocytes, monocytes, tissue macrophages and epithelial cells. Thus, the interaction of LFA-1 with its counter-receptor ICAM-1 covers a broad range of immune cell contacts and functions including antigen presentation, T-helper and B-lymphocyte responses, natural killing, and adherence of leukocytes to epithelial cells [2].

The $beta_3$ integrins are expressed on platelets, endothelium, polymorphonucleates and monocytes. All bind fibrinogen and von Willebrand factor, and some also bind fibronectin and vitronectin. The member known as gp IIb/IIIa is the major integrin of platelets where it binds soluble fibrinogen and von Willebrand factor. These molecules work as multivalent ligands, that is, they bind to other gp IIb/IIIa receptors on other platelets promoting platelet

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aggregation, or to receptors on endothelial cells or leukocytes, causing adherence of platelets to these cells.

A number of integrins are formed with newly discovered beta chains and are not as yet grouped in any subfamily. Some of them are expressed on epithelium ($\alpha_6\beta_4$) or on endothelium ($\alpha_v\beta_5$) and play a role in cell attachment to matrix substrate.

Selectin family

The selectins are three surface glycoproteins characterized by a similar structure consisting of an N-terminal lectin-like domain, an epidermal growth factor (EGF) repeat, and a variable number of modules homologous to complement binding proteins [11]. The term selectin highlights the functional importance of the lectin domain and the selectivity of distribution and function of these molecules. The lectin domain plays a crucial role, of course, containing the site that binds to specific carbohydrate counter-receptors, but the EGF domain is likewise essential in mediating adhesion. The role of the complement binding-like modules is less defined, possibly involving the mechanical support that keeps the lectin and EGF domains away from the cell surface. The selectins are named according to the cell type on which they were originally identified: E-selectin (endothelial cells), P-selectin (platelets) and L-selectin (leukocytes). E-selectin expression is restricted to endothelial cells activated by endotoxin or cytokines [12]. P-selectin is found in both platelets and endothelial cells, where it is stored in alpha and Weibel-Palade granules, respectively, in the resting state [13], and is rapidly redistributed to the cell surface upon activation by thrombin, histamine or other mediators [14]. L-selectin is constitutively expressed on lymphocytes, neutrophils and monocytes and is rapidly shed from their surface after cell activation. All three selectins bind to one or more carbohydrate ligands, mainly to sialyl-Lewis x and other fucosylated carbohydrates [15]. Counter-receptors of E-selectin are found on granulocytes, monocytes and a subpopulation of memory T-lymphocytes; P-selectin ligand is similarly distributed on leukocytes, and L-selectin ligand is expressed on endothelial cells [15]. Thus, all three selectins support the adhesion of leukocytes to endothelium, a necessary step for leukocyte migration in inflamed tissue. The adhesive reaction mediated by selectins is very quick, involving small carbohydrate epitopes [16], and is of low affinity. It is now clear that selectins play a role in the first step of leukocyte migration, which consists of leukocyte rolling on the endothelial surface and precedes the firm adherence mediated by integrins [17]. In addition to being essential for leukocyte recruitment in inflamed tissue, selectins regulate lymphocyte recirculation, that is, the passage of lymphocytes from blood to tissue or lymph node and then back to blood. Thus L-selectin functions as a "homing receptor" (this term refers to receptors on lymphocytes, while the counter-receptors on endothelium are called "addressins") for peripheral lymph nodes, while E-selectin functions as addressin for a subtype of memory lymphocytes [3].

Immunoglobulin superfamily

As previously discussed, the adhesion molecules of this group are ligands of integrin receptors.

ICAM-1 and ICAM-2 (intercellular adhesion molecule-1 and -2), the counter-receptors of $beta_2$ integrins, are closely related in structure and function but differ in the number of Ig domains (5 and 2, respectively). ICAM-2 is constitutively expressed on endothelial cells and its expression is not modified by stimulation; in contrast, the constitutive expression of ICAM-1 on endothelial cells varies in different vascular districts and is usually low. Endothelial expression of ICAM-1, however, increases several times after cell activation with IL-1, TNF or γ-interferon [18]. In addition to endothelial cells, other cell types express ICAM-1 after exposure to inflammatory stimuli, including leukocytes, epithelial cells, dendritic cells, and fibroblasts. Thus the interaction between $beta_2$ integrins (mainly LFA-1) and ICAM-1 regulates leukocyte migration, the immune responses that depend on adhesive contact between immune cells, and the binding of leukocytes to epithelial cells and fibroblasts [2]. Circulating forms of ICAM-1 were recently detected and their biological functions are currently under investigation [19].

VCAM-1 (vascular cell adhesion molecule-1) is another adhesion molecule of the immunoglobulin superfamily that contains seven Ig domains. VCAM-1 is induced by activation on endothelial cells and supports the adhesion to endothelium of memory lymphocytes [20] and other leukocytes expressing its $beta_1$ integrin counter-receptor VLA-4, that is, monocytes, basophils and eosinophils. VCAM-1 is also expressed on several non-vascular cell types, including dendritic cells in lymph node and skin, bone marrow stromal cells and synovial cells [11].

Chemokines

The chemokines are a superfamily of polypeptides that are characterized by the presence of four conserved cysteine (C) residues. Two subfamilies are distinguished according to whether another amino acid (X) is interposed between the first two C residues (alpha or C-X-C subfamily), or whether these two C residues are adjacent (beta or C-C subfamily). At present, fifteen related chemokines have been described that are secreted by different cell types such as platelets, white blood cells, endothelial and epithelial cells, macrophages, and fibroblasts [21]. All chemokines are chemoattractants, that is, they attract selected cell types expressing receptors specific for them. Representative members of the two subfamilies are interleukin-8 (C-X-C type) and RANTES (C-C type), both of which play a relevant role in inflammation by participating in leukocyte recruitment. These chemokines function through a mechanism known as haptotaxis, which makes them more similar to adhesion molecules than to classic chemotactic factors. In fact, they do not diffuse freely away from their site of production but bind to activated endothelium or extracellular matrix, so that leukocyte migration occurs along the gradient of chemokine affixed to the substratum. Leukocyte receptors for chemokines are members of the G-protein-coupled receptors and not only direct migration, but also activate integrin adhesiveness and stimulate degranulation and the respiratory burst. Their cellular distribution determines the responding cell; thus, the IL-8 receptor is restricted to neutrophils, while the RANTES receptor is expressed on monocytes, basophils, eosinophils, and a subset of T lymphocytes with high reactivity to recall antigens [22].

Renal expression of adhesion molecules

Adhesion molecules in cultured renal cells

Glomerular epithelial cells. Rat glomerular epithelial cells (GEC) in culture constitutively express the $beta_1$ integrins VLA-2 and VLA-3. These integrins mediate GEC adhesion to plastic wells coated with extracellular matrix proteins (collagen I and IV, laminin and fibronectin), and the adhesion is divalent cation dependent, a function characteristic of integrins [23]. These

observations suggest that beta$_1$ integrins regulate *in vivo* GEC attachment to glomerular basement membrane and play a role in maintaining the normal architecture of the glomerular capillary wall. It is of interest that the adhesion of rat GEC to collagen induces responsiveness to epithelial growth factors and activates phospholipase C [24], suggesting that the interaction of integrins with extracellular matrix regulates not only adhesion, but also GEC proliferation. Caution should be used, however, when extrapolating these data to the *in vivo* condition, in which GEC assume a unique shape with only the foot processes left in contact with the basement membrane. Recent studies, in fact, have shown that VLA-2 is not espressed in normal rat glomeruli, nor in GEC newly isolated from glomeruli, but is synthesized *ex novo* by GEC after several passages in culture, a phenomenon that probably depends on loss of differentiation [25]. Accordingly, immunoprecipitation of GEC lysates with anti-alpha chain antibodies has revealed only α_3, that is, the alpha chain of VLA-3 [26]. VLA_3 therefore appears to be the major or sole GEC integrin receptor.

Glomerular endothelial cells. In vitro rat glomerular endothelial cells express beta$_1$ integrins that mediate adhesion to components of the glomerular basement membrane; in particular, in culture conditions VLA-5 is the major fibronectin receptor, VLA-3 binds type I collagen, and VLA-1 participates in adhesion to laminin [27].

Mesangial cells. Human mesangial cells in culture use the VLA-5 integrin receptor to bind to wells and beads coated with fibronectin; interestingly, this VLA-5-mediated adhesion activates phagocytosis of fibronectin-coated beads. [28]. Recently, mRNA transcripts of integrin alpha chains 1, 2, 3 and 5 have been found in cultured human and rat mesangial cells, and immunofluorescence analysis has revealed that their protein products are concentrated into focal adhesions, supporting their functional relevance [29].

Murine mesangial cells express low constitutive levels of ICAM-1 and ICAM-1 mRNA transcripts. The expression of ICAM-1 increases markedly after stimulation with INF-γ and TNF, and mesangial cells become capable of adhering to T lymphocytes and of presenting antigen; anti-ICAM-1 mAb inhibits both adhesion and antigen presentation [30].

Tubular cells. Beta$_1$ integrins are expressed in primary cultures of human renal tubular epithelial cells and contribute to cell attachment to substratum; more precisely, VLA-5 binds tubular cells to fibronectin, and VLA-2 and VLA-6 bind them to laminin [31]. In addition to the ventral surface of cells, VLA-2 has also been detected at intercellular borders, where it may function during cell-cell interaction [32].

ICAM-1 was found to be constitutively expressed in over 90% of human proximal tubular cells in an established primary culture. ICAM-1 expression was up-regulated by supernatants from mixed lymphocyte reaction and by recombinant cytokines, especially INF-γ, TNF-α and IL-1 [33]. Adhesion of activated T lymphocytes to human tubular cells *in vitro* is inhibited by antibody to ICAM-1 [34]. These findings suggest that ICAM-1 plays a critical role in mediating lymphocyte adhesion to tubular cells.

VCAM-1 and its mRNA transcript are expressed in unstimulated human tubular cells in primary culture, and their expression is enhanced by stimulation with TNF-α [35]. Similar constitutive VCAM-1 expression and up-regulation by cytokines occurs in primary culture of mouse tubular cells; adhesion of homologous T cells and monocytes layered on stimulated murine tubular cells is inhibited by anti-VCAM-1 antibody [36].

Table 1. Expression of adhesion molecules in normal human kidney

Adhesion molecule	Renal expression		
	Glomeruli	Tubules	Interstitium and vessels
Integrins			
VLA-1	Mesangium, Bowman's capsule	All tubular cells	NE
VLA-2	Endothelium	Distal tubule	NE
VLA-3	Mesangium Visceral epithelium Parietal epithelium Endothelium	Distal tubule	NE
VLA-5	Mesangium Endothelium	NE	NE
VLA-6	NE	All tubules	NE
LFA-1	Monocyte/macrophage	NE	Monocyte/ macrophage
Ig superfamily			
ICAM-1	Endothelium	NE	Vascular endothelium
VCAM-1	Parietal epithelium	Proximal tubule	NE
Selectins			
E-selectin	NE	NE	Peritubular capillaries

NE is not expressed.

RANTES mRNA transcript and its protein product are expressed in a culture line of proximal tubular cells of murine origin, and the transcript level rises in response to stimulation with TNF-α and IL-1α [37]. IL-8 has also been detected in human renal epithelium [38]. These observations suggest that chemokines released *in vivo* by inflammation-damaged tubular epithelial cells may provoke or enhance the infiltration of leukocytes in renal interstitium.

Adhesion molecules in normal kidney

The expression of adhesion molecules in normal human kidney is summarized in Table 1.

Integrins. With the help of immunoelectron microscopy a fibronectin receptor of the integrin beta$_1$ subfamily was localized early in human kidney, in the membranes of glomerular cells facing the mesangial matrix and the glomerular basement membrane [39]. Successive studies using monoclonal antibodies specific to single beta$_1$ integrins have identified VLA-3 (a receptor that binds fibronectin, laminin and collagen) as the predominant VLA in the human glomerulus, where it is located in the mesangium, Bowman's capsule and on the visceral epithelial and endothelial cell surfaces in contact with the basement membrane. The predominantly basal distribution of VLA-3 suggests that it has a role in the attachment of glomerular endothelial and visceral epithelial cells to glomerular basement membrane [28]. In other studies, VLA-1 (receptor for laminin and collagen) was detected in mesangium and Bowman's capsule, VLA-2 (receptor for laminin) in glomerular endothelial cells, and VLA-5 (receptor for fibronectin) in mesangium and glomerular endothelial cells [32, 40]. Beta$_1$ integrins have also been isolated in proximal tubular cells, where they are strictly basally confined, and in distal tubule and ascending Henle's loop, where they are distributed both in a sharp linear fashion basally and diffusely throughout the rest of the cell membrane [41]. MoAbs directed against specific alpha chains have demonstrated that the alpha$_6$ chain, along with its

corresponding beta$_1$ integrin VLA-6 (laminin receptor), is confined basally in all renal tubules, presumably anchoring epithelial cells to the tubular basement membrane [42, 43], while VLA-2 and VLA-3 are restricted to the distal nephron [32]. VLA-1 (collagen receptor) is also expressed on the basal aspect of all tubular cells [44].

In normal kidneys LFA-1 expression is confined to isolated cells throughout the interstitium and in the glomeruli. In the latter, LFA-1 expressing cells number 1 to 10 per section and have been identified by immunostaining with specific antibodies as monocytes or migrating macrophages [34].

Immunoglobulin superfamily. The expression of ICAM-1 in normal human kidney has been the object of several studies [34, 35, 45, 46]. All agree that ICAM-1 is constitutively expressed in renal vascular endothelium, usually more intensely in interstitial capillaries and venules than in arterial vessels and glomerular capillaries. Most investigators have found no evidence of ICAM-1 expression in the mesangium, visceral glomerular epithelium, renal tubules; however, weak mesangial expression has also been described [47].

VCAM-1 is normally expressed on some parietal epithelial cells in Bowman's capsule and parts of the proximal tubule, but it is not constitutively expressed on visceral epithelium or endothelium [35, 48]. It is interesting that VCAM-1 distribution in normal kidney is complementary to that of the other immunoglobulin ligand of integrin receptors, ICAM-1, suggesting some complementation of roles in renal pathophysiological conditions characterized by the engagement of leukocytes.

Selectins. It has been reported that E-selectin is not detectable in normal human kidney [35], or that it is occasionally expressed in isolated peritubular capillaries [49]. The carbohydrate ligand of L-selectin, sLe^x, has been shown in small amounts in normal rat glomeruli [50].

Chemokines. The only study on distribution of RANTES in "normal" human kidney has been performed in renal biopsy samples of transplanted kidneys taken one hour after vascular anastomosis during transplantation surgery; in these kidneys neither RANTES nor its mRNA transcript were expressed [51].

Table 2. Adhesion molecules in renal disease

Adhesion molecules	Changes in renal disease (interested cell or site)
Intergrins	
β_1 integrins	δ Heymann nephritis (podocyte)
	δ "new" models of NTN (podocyte)
	δ anti-thymocyte serum nephritis (glomeruli)
	δ ischemic/toxic acute renal failure (tubular cells)
	↓ **membranous nephropathy** (glomerular capillary)
	↑ **IgA nephropathy** (mesangium)
β_2 integrins	↑ **hemodialysis** (peripheral leukocytes)
α_3, α_6, α_v subunits	δ **renal carcinoma** (tumoral cells)
Ig superfamily	
ICAM-1	↑ "classic" NTN (glomerular and interstitial capillary endothelium, interstitial resident cells)
	↑ murine lupus nephritis (mesangium, proximal tubule, vascular endothelium)
	↑ **focal glomerulosclerosis** (mesangium)
	↑ **extracapillary glomerulonephritis** (cellular crescents, tubules)
	↑ **human lupus nephritis** (glomeruli, tubules)
	↓ **membranous nephropathy** (glomerular capillary endothelium)
	↑ **"active" nephritides** (tubules)
	↑ **acute and chronic rejection** (tubules)
	↓ **hemodialysis** (peripheral leukocytes)
VCAM-1	↑ "classic" NTN (glomerular capillary endothelium)
	↑ murine lupus nephritis (mesangium, proximal tubule)
	↑ **glomerulonephritides and noninflammatory glomerular diseases (diabetes, gout and amyloid nephropathy)** (proximal tubule)
	↑ **acute rejection** (vascular endothelium, tubules, dendritic interstitial cells)
	↑ **cryoglobulinemia** (glomerular capillary wall)
Chemokines	
RANTES	↑ **acute rejection** (tubular cells, peritubular capillary endothelium)
Selectins	
E-selectin	↑ "classic" NTN (glomerular capillary endothelium)
L-selectin	↓ **hemodialysis** (peripheral leukocytes)

Symbols are: ↑ upregulation, ↓ downregulation, δ dysfunction. Changes found in human diseases are indicated in bold.

Pathogenic role of adhesion molecules in renal disease

The changes in adhesion molecules associated with renal disease are summarized in Table 2.

Adhesion molecules in nephritis

Experimental models: Heymann nephritis and nephrotoxic serum nephritis. In classic passive Heymann nephritis (HN), proteinuria occurs when anti-Fx1A antibodies raised against a proximal tubular antigen cross react with antigens on glomerular visceral epithelial cells (GEC) and gradually accumulate in the subepithelial space, leading to activation of complement and proteinuria in four to five days [52]. Proteinuria, however, can also be induced in 24 hours by injecting F(ab)$_2$ or F(ab)′ fragments of anti-Fx1A antibody, which do not activate complement [53]. This rapid-onset, complement-independent proteinuria is transient and associated with fine subepithelial deposits and a characteristic morphologic alteration consisting of effacement of GEC foot processes. Clearly the lesion produced by F(ab) fragments differs from that of classic HN. Adler and Chen recently showed that anti-Fx1A antibody recognizes the VLA-3 integrin receptor on GEC in culture. In their study, anti-Fx1A inhibited the adhesion of GEC to several substrata and produced reversible cell detachment and "rounding up" when added to adherent cells [54]. These results suggest that the binding of anti-Fx1A or its F(ab) fragments to the main integrin receptor of podocytes may alter the normal adhesion of GEC to GBM and cause cell detachment, which is responsible for altered glomerular permeability and proteinuria. The different disease courses caused by whole antibody and F(ab) fragments depend on differences in size and ability to activate complement. Due to their small size, F(ab) fragments are able to accumulate rapidly in the subepithelial space and in a short time produce a transient proteinuria, transient because it is not sustained by the inflammatory cascade initiated by complement activation.

Nephrotoxic serum nephritis (NTN) is a model of nephritis induced in rat by injecting heterologous anti-kidney antiserum. In classic NTN renal injury is both complement- and leukocyte-dependent and is caused by antibodies directed against antigens in the GBM. In the last few years, however, it has become clear that

nephrotoxic sera are polyreactive, that is, they contain antibodies directed against several cell-surface antigens in addition to the classic GBM antigens [55]. Furthermore, it has been shown that some of these antibodies can cause proteinuria directly, in the absence of complement activation or inflammatory cell infiltrates. Several new models of NTN have thus been designed [56, 57] that are clearly different from classic inflammatory NTN. In these models of NTN a characteristic morphological correlate of proteinuria is effacement and detachment of podocytes from GBM, so that it is reasonable to speculate that antibodies interfere with normal podocyte-GBM adhesion. Early evidence that podocytes might be the target of antiserum in complement-independent NTN was given by the demonstration that a monoclonal antibody against the epithelial surface antigen SGP-115/107 can directly induce both proteinuria and the characteristic structural alterations [58]. Recently, O'Meara and coworkers have shown that a non complement-binding anti-rat nephrotoxic serum reacts with surface antigens of GEC. This antiserum immunoprecipitates two proteins from GEC that are co-precipitated by a monoclonal antibody identifying the $beta_1$ integrin receptor for fibronectin. In addition, in culture the antiserum inhibits adhesion of GEC to collagen, laminin and fibronectin, and prevents GEC spreading on a variety of matrix proteins [59]. These findings suggest that anti-integrin antibodies disrupt GEC anchorage to GBM and thus cause detachment of foot processes and alteration of glomerular capillary permeability.

In summary, modifications of two classic models of nephritis have led to the development of models of antibody-dependent, complement-independent renal injury characterized by rapid-onset proteinuria, alterations of podocyte morphology (effacement of foot processes), and detachment of epithelial cells from the basement membrane. Interference with the normal anchorage of GEC to GBM caused by antibodies to integrin receptors on podocytes seems to be the common pathogenic mechanism in these models. It is reasonable to speculate that similar interferences with integrin function, caused by toxic substances or circulating factors, may play a pathogenic role in other models of non-inflammatory proteinuria, for example, puromycin nephrosis or human minimal change nephropathy.

Recent studies have shown that leukocyte adhesion molecules and their endothelial ligands also play a fundamental pathogenic role in classic NTN caused by anti-GBM antibodies. Infusion of anti-GBM antibodies, in fact, is followed by up-regulation of E-selectin, ICAM-1 and VCAM-1 on glomerular capillary endothelium. Leukocyte recruitment in glomeruli and proteinuria is inhibited by treating rats with antibodies against ICAM-1 and VCAM-1, and their counter-receptors LFA-1, Mac-1 and VLA-4. Protection is also afforded by anti-TNF-α antibodies, suggesting that this cytokine, perhaps released locally, is responsible for up-regulation of endothelial adhesion molecules [60–62]. In the early phase of anti-GBM nephritis, ICAM-1 is strongly up-regulated in the endothelium of interstitial capillaries as well and is expressed *de novo* in interstitial resident cells. ICAM-1 therefore appears to direct migration and localization of interstitial leukocytes [63].

Other models of nephritis. Wuthrich et al studied the renal expression of ICAM-1 and VCAM-1 in murine models of lupus nephritis. With the aid of immunoperoxidase staining they showed a strong up-regulation of ICAM-1, in particular in the brush border of proximal tubules, in the mesangium and in the endothelium of large vessels. Northern analysis revealed a two- to fivefold increase in the levels of ICAM-1 transcripts in the kidney of nephritic mice [64]. Like ICAM-1, VCAM-1 was up-regulated in cortical tubular and mesangial cells, but the tubular expression of VCAM-1 was more focal, being localized in tubules adjacent to mononuclear infiltrates. Adherence assays on kidney sections from autoimmune rats showed an increased adhesiveness of T cell and macrophage cell lines and of lymph node cells that was inhibited by monoclonal antibodies targeting the ICAM-1 and VCAM-1 molecules [36]. These results indicate that enhanced ICAM-1 and VCAM-1 expression confers increased adhesiveness on the renal parenchyma in lupus nephritis and serves as a pathway by which inflammatory cells adhere to renal tissue and promote renal injury.

Injection of anti-thymocyte serum produces an acute form of glomerulonephritis in the rat characterized by transient mesangiolysis, followed by mesangial cell activation and mesangial matrix synthesis that is promoted by increased local activity of TGF-β [65]. Recent studies have shown that the increased matrix deposition in this glomerulonephritis is associated with over-expression of glomerular $beta_1$ integrins VLA-1 and VLA-5 and reduced expression of VLA-3. In *in vitro* studies, normal glomeruli treated with TGF-β showed changes in integrin expression mimicking those occurring *in vivo*. These results suggest that the proliferative effect of TGF-β is mediated by altered $beta_1$ integrin expression [66].

Human nephritis. Seron et al studied the expression of VCAM-1 in renal biopsies from patients with interstitial nephritis due to NSAID, systemic vasculitis with crescentic nephritis, and other forms of inflammatory and non-inflammatory glomerular disease (IgA, membranous, minimal change, diabetes, lupus, gout and amyloid nephropathy). Proximal tubular expression of VCAM-1 was elevated in all such disorders, but the increase was especially marked in vasculitis and interstitial nephritis. VCAM-1 expression was positively correlated with the number of activated (transferrin-receptor positive) leukocytes infiltrating the interstitium. Surprisingly, VCAM-1 was never identified on vascular endothelium [48]. Up-regulation of VCAM-1 was also found in different forms of glomerulonephritis by Bruijn and Dinklo [67]. These authors documented increased expression on proximal tubules and on the endothelium of large interstitial vessels, but not on interstitial capillaries, in all the diseases studied (membranous and membranoproliferative glomerulonephritis, IgA nephropathy, lupus nephritis, cryoglobulinemia, Wegener's disease). Particularly strong expression of VCAM-1 in the glomerular capillary wall was observed in cryoglobulinemia. These findings suggest that VCAM-1 plays a pathogenic role in several renal diseases by promoting an interaction between inflammatory and tubular epithelial cells. Such interaction may be necessary for the mononuclear cell cytotoxic attack, as well as for the tubular cell accessory function as antigen presenting cell.

The author and his coworkers have studied the expression of ICAM-1 in primary focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), necrotizing glomerulonephritis (NGN) with crescent formation, and lupus nephropathy. We have shown intense *de novo* expression of ICAM-1 on mesangial cells in FSGS, with focal and segmental distribution, but no up-regulation of the molecule in MN [43]. While the pathogenic role of ICAM-1 in FSGS remains to be established, the increased mesangial expression of this molecule clearly indicates activation of mesangial cells and suggests an inflammatory step in the progression of

the disease. Interestingly, we recently found that ICAM-1 is expressed *de novo* in the mesangium of patients with minimal change disease, similar to what occurs in patients with FSGS (Dal Canton and Meyrier, unpublished observations), which supports the contention that the two diseases are but two sides of the same coin. We also discovered abnormal expression of ICAM-1 in the glomeruli of patients with crescentic glomerulonephritis. In fact, ICAM-1 was strongly expressed in cellular crescents, a finding that suggests a role for this molecule in recruiting macrophages in the early stage of the disease [68]. Increased glomerular expression was a marker of activity in lupus nephritis [69]. Other studies have shown altered renal expression of ICAM-1 in different forms of glomerulonephritis. Lhotta et al observed increased glomerular expression in early cases of rapidly progressive glomerulonephritis and in lupus nephritis, while the expression was reduced in advanced rapidly progressive glomerulonephritis and membranous nephropathy. Increased ICAM-1 expression was also detected on tubular cells in rapidly progressive glomerulonephritis and membranoproliferative glomerulonephritis [45]. Tubular expression of ICAM-1 was studied by Chow et al in renal biopsy specimens from a range of non-immune renal diseases and glomerulonephritides. Increased tubular expression of ICAM-1 was found on undamaged tubules in glomerulonephritis and this showed a strong correlation with disease activity [70]. In summary, ICAM-1 seems to play a role in promoting glomerular recruitment of leukocytes and extracapillary proliferation in exudative and crescentic glomerulonephritis, respectively; in addition, in several other forms of nephritis, *de novo* expression of ICAM-1 on tubular cells confers them a stickiness for adhesive contact with infiltrating leukocytes whose toxic attack may thus be facilitated.

Baraldi and coworkers studied the expression of integrin VLA-3 in renal biopsy specimens from patients with nephrotic syndrome due to membranous nephropathy (MN), minimal change disease and lupus nephritis [71]. It is noteworthy that the normal linear distribution of VLA-3 along the glomerular capillary loop was altered in membranous nephropathy but not in the other diseases, indicating that changes in MN were not an aspecific effect of proteinuria. In stages I and II of MN, VLA-3 distribution showed an irregular, trabecular pattern; in addition, in stage III a segmental loss of VLA-3 was detected [71]. These observations suggest that in human MN, as in its experimental counterpart (Heymann nephritis), a disruption of the normal interaction between VLA-3 and its GBM ligand occurs.

Recently, the expression of fibronectin receptor (assumed to be an integrin) was found to be elevated in the mesangium of patients with IgA nephropathy. This increase was correlated with the degree of histological glomerular damage and proteinuria [72]. The pathophysiological relevance of this observation is not clear, but one may speculate that up-regulation of the fibronectin receptor plays a role in the activation of mesangial cells that is the hallmark of this disease.

Adhesion molecules in renal transplant

Pathogenic role of adhesion molecules in graft rejection. Cell-mediated rejection is an immune reaction characterized by the migration of mononuclear cells into renal tissue and by an effector response driven by the interaction between MHC molecules on donor cells and TCR on recipient T cells. Since both adherence of leukocytes to endothelium (which is crucial for leukocyte migration) and stabilization of the TCR-antigen interaction (necessary for full T cell activation) are mediated by adhesion molecules, it is logical to expect that adhesion molecules play an important pathogenic role in rejection. Until now the interest of investigators has been mainly limited to evaluating renal expression of ICAM-1 in acute and chronic rejection [34, 35, 46, 49, 73–75]. All agree that graft rejection is accompanied by *de novo* expression of ICAM-1 on renal tubular cells. Such expression is predominant in the proximal tubules and has a focal distribution occurring in 15 to 40% of tubules. It is paralleled by up-regulation of HLA class II antigens and is roughly proportional to the level of leukocyte infiltration. The *de novo* tubular expression of ICAM-1, however, is not specific to graft rejection, since it has also been found in non-rejecting kidneys [49] and may depend on ischemic damage during organ retrieval [46]. Increased ICAM-1 expression on renal vascular endothelium has occasionally been described during rejection [46], but this finding is inconsistent, mainly because ICAM-1 is constitutively and diffusely expressed on normal renal endothelium as well [34, 49]. In summary, these studies suggest that in renal graft rejection significant *de novo* ICAM-1 expression on tubules supports the contact between tubular cells and infiltrating leukocytes. In this way, both the antigen presentation by tubular cells (in the context of class II MHC molecules) and the cytotoxic attack of effector cells are facilitated. A role for ICAM-1 in the recruitment of leukocytes into renal tissue has not definitely been proven, and renal expression of ICAM-1 cannot be used to discriminate rejection from other causes of acute graft dysfunction. An attempt has been made to utilize serum levels of circulating ICAM-1 or ICAM-1 expression on renal tubular cells present in urinary sediment as diagnostic indicators of acute rejection, but with poor success [75, 76]. In fact, circulating ICAM-1 concentration increases not only during rejection but also during cytomegalovirus infection, and cells expressing ICAM-1 are found in the urine of both rejecting and non-rejecting patients.

A number of studies have recently investigated the expression of other adhesion molecules in renal graft rejection. In acute rejection *de novo* expression of VCAM-1 is induced on renal vascular endothelium, and the expression of this molecule is up-regulated in the tubules [35, 49, 73, 74]. *De novo* VCAM-1 expression is particularly pronounced on the endothelium of muscular arteries showing features of acute vascular rejection, that is, endothelial separation from the underlying intima and subendothelial infiltration of mononuclear cells. In acute vascular rejection VCAM-1 is also found on a distinct population of dendritic cells within interstitial lymphoid aggregates, and focally on mesangial cells [77]. These results suggest that VCAM-1 plays a double pathogenic role in renal graft rejection by (a) potentiating the interaction between tubular cells and effector cells, and (b) promoting leukocyte migration into renal tissue. *De novo* induced expression on renal endothelium is particularly relevant and seems to be a distinctive feature of rejection that is useful for differentiating acute rejection from other causes of graft dysfunction, for example, cyclosporine toxicity [73].

Only a few studies have examined E-selectin in renal allograft rejection in humans, and no change in its expression was detected [49, 74]. E-selectin, however, is induced early and transiently in inflammatory response so that grafts may have been biopsied after the level of E-selectin had declined. A role for L-selectin and its sialyl Lewis^x ligand in determining the site of lymphocyte extravasation into the graft has recently been shown in a rat model of acute renal graft rejection [50].

The role of the RANTES chemokine in cell-mediated transplant rejection was recently defined in elegant studies by Pattison et al [49]. These authors demonstrated that during rejection both RANTES and RANTES mRNA are expressed by infiltrating mononuclear cells and by renal tubular cells. In addition, they localized RANTES protein (but not mRNA) on the endothelium of peritubular capillaries and showed that RANTES receptors are expressed on this endothelium. These results allow construction of a model of the central role played by RANTES in transplant rejection. Macrophages in the graft produce cytokines that activate the expression of RANTES by renal tubular epithelium. RANTES released by tubular cells diffuses locally and binds to receptors on endothelial cells. After being planted on the endothelium RANTES works as an adhesion molecule, attracting T cells and monocytes to the site of inflammation, and once these cells have entered the interstitium they are driven to their target by the RANTES gradient expressed on tubular cells.

Anti-rejection therapy with anti-adhesion molecule mAbs. An anti-LFA-1 monoclonal antibody was first used successfully to prevent graft failure in children with HLA-mismatched bone marrow transplants [78]. The same antibody (murine mAb 25-3) was then used to treat seven subjects with acute renal graft rejection. Only one patient, who had the lowest increase in blood creatinine, reverted to pre-rejection graft function with 25-3 treatment alone. All the others required additional rescue therapy, so it was concluded that anti-LFA-1 is inefficient in reversing acute ongoing renal rejection. Treatment with 25-3 caused a reversible side effect (Quincke's edema) in only one patient and did not raise anti-murine antibodies in any case [79]. These results, while disappointing, do not exclude the possibility that the antibody may be more useful for preventing renal graft rejection (as in bone marrow transplantation) than for treating rejection episodes in course.

The effects of blocking ICAM-1 were first studied by Cosimi et al in cynomolgus monkeys [80]. These authors administered a murine anti-ICAM-1 mAb (R6.5) as the sole agent to prevent rejection in a group of animals and used R6.5 to treat ongoing acute rejection in another group maintained on cyclosporine. Treatment with the antibody significantly prolonged survival in the first group and reversed rejection in all animals of the second group. These results encouraged Haug et al to use the same antibody in a phase I trial in human renal allograft recipients. Eighteen patients who received allografts at high risk for delayed graft function received doses of R6.5 in order to maintain adequate serum levels (>10 μg/ml) for a two-week course. The patients treated with anti-ICAM-1 mAb had significantly fewer delayed graft function and rejection episodes than a group of patients who received the contralateral kidney from the same donor and conventional treatment. In addition, none of the anti-ICAM-1 treated patients developed primary non-function, in contrast with 3 out of 18 in the control group [81]. Therapy with R6.5 was well tolerated.

In conclusion, preliminary results indicate that anti-ICAM-1, but not anti-LFA-1 therapy, may be useful in treating acute renal graft rejection. Why blocking ICAM-1 is efficient but blocking its ligand is not remains unclear. A possible explanation is that anti-LFA-1 operates only on leukocytes, while anti-ICAM-1 can also act at the target cell level. Part of the benefit displayed by anti-ICAM-1, however, may result from a protective effect on reperfusion injury.

Effects of extracorporeal hemodialysis on leukocyte adhesion molecules

Hemodialysis causes a profound, transient neutropenia whose possible mechanisms include leukocyte aggregation, adhesion to endothelia, and pulmonary sequestration [82]. There is evidence that dialysis-related neutropenia depends on complement activation and generation of the complement fragments C3a and C5a, which can activate leukocytes [83]. Arnaout et al first demonstrated a rapid up-regulation of the granulocyte beta$_2$ integrin Mac-1 during dialysis with cellulose filters [84]. Interestingly, the time course of increased expression of this receptor coincided with the development of granulocytopenia and with the peak rise in plasma levels of the complement activation products C5a desArg and C3a desArg. C5a desArg (but not C3a desArg) induced a comparable increase in Mac-1 expression on normal granulocytes *in vitro* at concentrations similar to those measured *in vivo*. The overexpression of Mac-1 during cuprophane dialysis was confirmed by Alvarez et al, who also found that dialysis increased leukocyte expression of p 150,95, another beta$_2$ integrin, and down-regulated the expression of L-selectin. Similar phenotypic changes were reproduced *in vitro* by incubating neutrophils with activating agents. Kinetic studies showed that both the peak increase in integrin expression and the maximal drop in L-selectin expression occurred after 15 minutes of dialysis, coincident with the nadir in neutrophil count. Neither neutropenia nor the changes in leukocyte adhesion molecules took place when hemodialysis was performed with polyacrylonitrile membranes [82]. The ability of cuprophane membranes to activate serum directly was proved by Lundahl, Hed and Jacobson, who showed that serum preincubated with fragments of cuprophane membranes increased Mac-1 expression on normal leukocytes [85]. The causal relation between complement activation and changes in leukocyte adhesion molecules during hemodialysis was further confirmed by Himmelfarb et al [86], who utilized either first-use cellulose dialyzers (that activate complement) or reused dialyzers (that have minimal complement activating potential). First use dialyzers, but not the reused ones, caused a rapid, fourfold increase in Mac-1 and a twofold decrease in L-selectin. Stimulation of granulocytes *in vitro* with C5a reproduced the same changes seen *in vivo*. Taken together, these results demonstrate that dialysis with cellulosic membranes causes complement activation and generates complement products that can activate leukocytes. Leukocyte activation is associated with changes in the expression of adhesion receptors that are responsible for leukocyte sequestration and leukopenia. Himmelfarb et al also performed adhesion assays of leukocytes on monolayers of human umbilical endothelial cells [86]; the ability to adhere leukocytes with the "high Mac-1, low L-selectin" phenotype, harvested from patients during dialysis, was dramatically decreased. This intriguing finding may be explained by the rapid dialysis-induced transformation of granulocytes into a "refractory" post-activation state, in which shedding of L-selectin caused by activation [11] prevents adhesion. Preliminary results suggest that other changes in surface adhesion molecules caused by dialysis, such as reduced ICAM-1 expression [87], account for the loss of the adhesive capacity of leukocytes and thus may contribute to the increased susceptibility to infection of dialysis patients.

Another leukocyte dysfunction caused by hemodialysis is degranulation of peripheral blood neutrophils. Degranulation occurs mainly, but not exclusively, with cuprophane membranes and

is an untoward phenomenon that participates in the pathogenesis of the catabolic state and of the carpal-tunnel syndrome, which are frequently observed in dialysis patients [88, 89]. Recently, Cheung et al [89] have shown that products generated by the contact of plasma with the cuprophane membranes are responsible for degranulation. These products have not been precisely identified but are in part complement dependent, in part complement independent. In any case, they act on leukocytes through a common pathway that consists of $beta_2$ integrins. In fact, unlike normal leukocytes, those harvested from a patient with a congenital deficiency of $beta_2$ integrins do not degranulate when incubated with plasma and cuprophane membrane.

In summary, the present information indicates that adhesion molecules mediate leukocyte dysfunctions caused by hemodialysis. Therefore the effects of different dialysis membranes on leukocyte adhesion receptors should be taken into account as an important index of biocompatibility.

Adhesion molecules and other renal disorders

Acute renal failure. Obstruction of renal tubules by desquamated cells and debris plays an important pathogenic role in post-ischemic and nephrotoxic acute renal failure [90, 91]. Recent studies have shown that the majority of cells desquamated after renal insults are viable, suggesting that active cell aggregation may contribute to the development of tubular obstruction [92]. In addition, it has been found that oxidant stress to renal epithelia results in the disruption of focal contacts, the disappearance of talin from the basal cell surface (talin is a cytoskeleton protein connected with the intracellular part of integrin receptors), and a redistribution of the $alpha_3$ integrin subunit from its predominantly basal location to the apical cell surface. These changes are associated with decreased adhesion of tubular cells to wells coated with type IV collagen, laminin, fibronectin and vitronectin [93]. The ectopically expressed apical integrins, however, are functionally competent and capable of mediating cell-cell adhesion in cell suspensions [94]. Based on the above observations, the following scenario has been hypothesized for the development of tubular obstruction secondary to tubular cell insult: the loss of basolateral expression of integrins is responsible for the detachment of cells from the matrix, whereas the expression of integrin receptors on the apical surface accounts for facilitated cell-cell adhesion and eventual tubular obstruction [95] This hypothesis is supported by the observation that infusion of an integrin inhibitor in rats subjected to renal ischemia prevents a rise in intratubular pressure, the pathophysiological marker of tubular obstruction [94].

Renal cysts. Renal cyst formation derives from abnormal tubular cell proliferation and is accompanied by abnormalities in the synthesis of matrix by cyst-lining cells [96] and changes in epithelial polarity [97]. It is known that epithelial cells are responsible for the composition of their basement membranes, and the basement membrane in turn modulates cell movement, division, differentiation and polarity. As discussed previously, tubule cell-matrix interactions are mediated by adhesion receptors, mainly of the integrin family. It is not surprising, therefore, that preliminary results indicate that members of the $beta_1$ integrin family are involved in the dysregulation of the epithelial polarity that occurs in a model of *in vitro* cyst formation [98]. A role for attenuated expression of epithelial cell adhesion molecules has been suggested in a murine model of polycystic kidney disease [99].

Renal cell carcinoma. Integrin distribution has been studied in renal cell carcinomas of various grades of malignancy [100]. The $alpha_3$ subunit was detected in all tumors regardless of the grade of malignancy. This finding is interesting because renal cell carcinomas are believed to originate from both proximal and distal tubular cells, while in normal kidney $alpha_3$ expression is restricted to the distal tubule. The $alpha_6$ subunit was expressed in low-grade malignancies, in which it coaligned with basement membrane components expressed in the matrix. In contrast, the $alpha_6$ subunit was absent in G3 carcinomas, reflecting a complete disruption of cell-basement membrane interaction and, possibly, metastatic invasiveness. Noteworthy was the *de novo* expression of $alpha_v$ chain, which is not present in normal kidney and is correlated with the degree of malignancy. In summary, these results indicate that changes in integrin expression are associated with malignant transformation of cells in renal carcinoma and may play a role in their metastatic diffusion.

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Commentary

Tissue Factor in Crescentic Glomerulonephritis

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Studies performed over many years have shown that blood coagulation plays a pathogenic role in certain glomerular diseases and is particularly important in crescent formation.[1,2] Conspicuous accumulation of fibrin is seen in crescents, and often also within glomerular tufts, both in human diseases and experimental models. More important, prevention of crescent formation has been achieved in animal models by anticoagulation,[3] defibrination (with ancrod),[4-6] or fibrinolytic therapy with tissue plasminogen activator (t-PA).[7,8] Most of these results have been obtained in models of anti-glomerular basement membrane (anti-GBM) antibody nephritis, but defibrination has been shown to suppress the development of crescents in a model of serum sickness,[5] indicating that crescent formation depends on coagulation irrespective of the nature of the underlying glomerular disease.

The development of crescents requires severe damage to glomerular capillary walls, often with disruption of the basement membrane apparently due to leukocyte proteolytic enzymes,[9] resulting in passage of large plasma proteins, including fibrinogen, and of leukocytes and red cells into Bowman's space.[10,11] Fibrin-rich proteinaceous deposits are formed, often associated initially with adhesions of glomerular tufts to Bowman's capsule. During a period of several weeks, larger and increasingly cellular structures develop in and often obliterate Bowman's space, due mainly to accumulation of macrophages[10,12] and proliferation of epithelial cells.[11] Later, crescents are usually invaded by fibroblast like cells, associated with the deposition of collagen and other extracellular matrix components including hyaluronic acid and fibronectin,[11,13-16] leading to irreversible nephron loss. This sequence is not inevitable, however, and cellular crescents sometimes disappear completely without undergoing fibrosis.[17] Furthermore, even after many weeks, crescents in various stages of development can be seen. These considerations suggest that appropriate treatment of crescentic glomerulonephritis (if such could be found) might arrest further crescent formation and even lead to removal of nonfibrotic crescents.

Several investigations have dealt with the pathway of coagulation that leads to fibrin formation in Bowman's space. Some observations support the role of the intrinsic pathway (the contact system).[18-20] Although this system has come to be regarded as largely a test tube phenomenon, there are grounds for suspecting that it is activated within Bowman's space during crescent formation. Thus, it has been shown that the GBM, a negatively charged structure, can activate the contact system *in vitro*.[21] Presumably, released fragments of GBM trigger coagulation in Bowman's space.

Although the contact system may contribute to glomerular fibrin deposition in crescentic glomerulonephritis, considerable evidence has accumulated favoring the primary importance of the extrinsic pathway, triggered by activation of tissue factor, also known as tissue thromboplastin. Tissue factor is a transmembrane glycoprotein expressed constitutively in many cells, especially those not normally exposed to the circulation.[22] However, tissue factor expression can be induced in activated macrophages or endothelial cells. When blood is extravasated, circulating factor VII/VIIa combines with tissue factor to form an enzyme complex that activates factor X to Xa, which in turn generates a complex that converts prothrombin to thrombin.[23] Thrombin

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cleaves fibrinogen, yielding monomeric fibrin, which then polymerizes to form the fibrin clot. This is considered to be the usual pathway of coagulation *in vivo*, at sites of bleeding or thrombosis.[23,24]

Direct evidence for the essential role of tissue factor in glomerular fibrin deposition is provided by the report of Erlich et al in this issue of the journal.[25] They showed that administration of antibodies to tissue factor reduced glomerular tissue factor activity, largely prevented fibrin deposits in Bowman's space, and protected against renal functional impairment in rabbits developing anti-GBM nephritis. These effects were achieved without hemorrhagic complications. They also obtained evidence that tissue factor has effects other than interference with coagulation, as evidenced by decreased major histocompatibility complex class II expression in glomeruli and tubulointerstial sites in the rats given antibodies to tissue factor. The present work builds on a previous investigation by these authors, in which they demonstrated up-regulation of glomerular tissue factor activity in anti-GBM nephritis.[26] Initially, this resulted from up-regulation of tissue factor in intrinsic glomerular cells and, later, from synthesis by infiltrating macrophages. Urinary excretion of tissue factor was demonstrated, beginning early in the disease. In an earlier report, Wiggins and associates showed that membrane vesicles containing a procoagulant signal, probably tissue factor complexed with factor VII, are shed from glomerular epithelial cells in rabbit anti-GBM nephritis.[27]

In addition to factors that initiate coagulation, impairment of fibrinolytic mechanisms may contribute to glomerular fibrin accumulation. Thus, reduced glomerular fibrinolytic activity associated with decreased expression of t-PA and increased expression of plasminogen activator inhibitor type-1 (PAI-1) has been observed in experimental anti-GBM nephritis.[28,29] Nevertheless, Thomson et al[6] showed that, when rabbits with established anti-GBM nephritis were treated with the defibrinating agent ancrod, existing glomerular fibrin deposits disappeared, indicating that fibrin-clearing mechanisms were not entirely abolished.

Numerous potential mediators of injury in experimental crescentic glomerulonephritis have been described,[30] but the importance of many remains uncertain, either because the postulated role does not operate *in vivo* or because redundant mediators can compensate for their loss. The demonstration that several types of treatment that prevent fibrin formation interfere with crescent formation in experimental crescentic nephritis indicates that the coagulation process cannot be bypassed. This raises the possibility that interference with coagulation or enhancement of fibrinolysis might be beneficial in human crescentic glomerulonephritis, as discussed below.

There is substantial evidence from experimental models that macrophages are required for glomerular fibrin deposition and crescent formation. Early infiltration by macrophages in glomerular capillaries is seen in experimental anti-GBM nephritis, and this accounts for much of the tuft hypercellularity seen in these models.[31] This is sometimes associated with fibrin deposition in glomerular tufts.[32] Later, increasing numbers of macrophages accumulate in crescents and constitute a major component of these structures.[10,33] The administration of antibodies to the adhesion molecules LFA-1 and ICAM-1 to rats developing autoimmune anti-GBM nephritis has been shown to reduce macrophage infiltration in glomeruli and largely prevent fibrin deposition and crescent formation.[34] Other procedures that reduce glomerular macrophage accumulation[32] or antagonize macrophage cytokines, including antibodies to macrophages[35] or migration inhibitory factor,[36] have also been shown to protect against crescent formation. Early studies showed that infiltrating macrophages express procoagulant activity, probably due to tissue factor.[37–39] Although macrophages can produce injury through numerous mechanisms, the findings in the present report by Erlich et al[25] provide convincing evidence that an essential way in which macrophages promote crescent formation is through expression of tissue factor.

Evidence for a key pathogenic role of macrophage tissue factor expression has been obtained by Immamura et al[40] in another form of immunological injury characterized by fibrin deposition, namely, cutaneous delayed-type hypersensitivity (DTH). The characteristic induration of DTH reactions is known to be due to fibrin deposition,[41] and anticoagulation has been shown to reduce the severity of DTH reactions.[42] Immamura et al studied the effects of anti-tissue factor antibodies on DTH reactions produced by intradermal injections of purified protein derivative in bacille Calmette-Guérin-sensitized monkeys. They found that unmodified DTH reactions were characterized by fibrin deposition in a network pattern around macrophages that expressed tissue factor. Furthermore, they demonstrated that local injection of a monoclonal anti-tissue factor antibody together with PPD largely prevented fibrin deposition and induration and reduced leukocyte accumulation. The monoclonal antibody used is known to inhibit VII/VIIa-tissue factor interactions, and its effectiveness thus provides evidence that tissue factor is responsible for coagulation in DTH. The observa-

tions also relate to the possibility that a DTH component may contribute to glomerular damage in anti-GBM nephritis, through interaction of sensitized T cells with glomerular-bound antigen, a mechanism that is supported by other evidence.[2,43–48]

Although it is clear that fibrin deposits in Bowman's space are essential for the initiation of crescent formation, the events involved in the further development of crescents are obscure. It is probable that proinflammatory factors, such as thrombin, fibrin fragments, or various cytokines, and other mediators of inflammation or proliferation produced by macrophages or other cells within the clot provide stimuli for the formation of cellular crescents. The factors that contribute to the deposition of extracellular matrix components leading to fibrosis of crescents are also not entirely understood, but interactions between cell receptors and matrix are probably crucial.[14] Fibronectin may play a major role in sclerosis of crescents. Thus, local synthesis of distinctive embryonic isoforms of fibronectin has been demonstrated in crescents undergoing fibrosis.[16]

Experimental models may provide insights into pathogenic mechanisms, but their ultimate value has to be judged by whether they offer ways in which human diseases can be prevented or treated. Most of the experimental studies dealing with suppression of crescents have demonstrated preventive rather than therapeutic effects. Nevertheless, treatment of animals with ongoing crescentic glomerulonephritis by defibrination[6] or anti-adhesion molecules[34] has produced beneficial effects. Clearly, in human crescentic glomerulonephritis, prevention is not possible, so the question to be discussed is whether treatment of established disease is feasible.

There are reasons to assume that coagulation and macrophages are important in the pathogenesis of crescents in humans, as in the experimental models. Staining for fibrinogen/fibrin and fibronectin is found in early crescents in human glomerulonephritis,[17] and macrophage infiltration of glomeruli has been demonstrated.[12] Furthermore, there is evidence that macrophage-derived tissue factor is responsible for glomerular fibrin deposition in human glomerulonephritis.[49] These considerations suggest that prevention or elimination of glomerular fibrin deposition might be beneficial. Based on knowledge gained from experimental anti-GBM nephritis, ways that can be considered include anticoagulation,[3] defibrination,[4–6] enhancement of fibrinolysis,[7] or possibly interference with infiltration or activation of macrophages.[34–36]

The safety and effectiveness of anticoagulation in experimental models has been variable, depending on the agent used and the dosage given. Administration of warfarin to rabbits resulted in striking reduction of fibrin deposition and crescent formation in experimental anti-GBM nephritis, but the doses required were so high that many animals died of hemorrhage.[3] Heparin has had variable effects in experimental models of anti-GBM nephritis. In some reports, administration of heparin caused reduction in fibrin deposition and crescents[4,50,51] but mainly when given in doses sufficient to produce extreme prolongation of clotting times. In other experiments, heparin was totally ineffective.[52,53] Variations in experimental protocols and severity of the disease may account for some of the differences. Furthermore, there are reasons for concluding that heparin may not be the ideal anticoagulant, as it acts only indirectly as an anti-thrombin agent, requiring antithrombin III as a cofactor, and because its effect is readily inactivated by plasma proteins.[54] In addition, heparin is not effective against thrombin bound to the fibrin clot.[54]

Agents that have been effective in reducing fibrin deposits and crescent formation in experimental models without producing hemorrhagic complications include ancrod,[4] antibodies to tissue factor or migration inhibitory factor,[36] and t-PA.[7] It should be noted, however, that therapeutic use of t-PA in man has been associated with bleeding complications.[55]

A few crescents can be seen in many human glomerular diseases, but extensive crescent formation (involving >50% of glomeruli) is largely confined to a small group of conditions, the most important of which are anti-GBM nephritis and pauci-immune necrotizing and crescentic glomerulonephritis.[17,56] The latter condition can be primary (apparently restricted to the kidney) or associated with Wegener's granulomatosis or microscopic polyangiitis (polyarteritis).[57,58] Major advances in the diagnosis of these conditions has come from the development of sensitive and specific tests for circulating pathogenic anti-GBM antibodies (against the α3 chain of the NC1 domain of type IV collagen) and for two types of anti-neutrophil cytoplasmic antibodies (ANCA), against proteinase 3 and myeloperoxidase, which are found in nearly all patients with pauci-immune necrotizing and crescentic glomerulonephritis and rarely in unrelated conditions.[56,57] The appropriate use of serological tests for anti-neutrophil cytoplasmic antibodies and anti-GBM antibodies facilitates rapid diagnosis of these conditions in patients who present with the features of rapidly progressive glomerulonephritis. As noted earlier, nearly all of the experimental studies demonstrating protective effects have been performed in models of anti-GBM

nephritis. There is at present no satisfactory model of anti-neutrophil cytoplasmic antibodies associated pauci-immune necrotizing and crescentic glomerulonephritis, which might permit evaluation of pathogenic mechanisms in this condition.

A major problem in performing therapeutic trials in humans with anti-GBM nephritis or pauci-immune necrotizing and crescentic glomerulonephritis is that these diseases are usually rapidly progressive, with the result that irreversible renal damage has often developed by the time diagnosis is established.[30] In fact, this is usually the case in anti-GBM nephritis. In pauci-immune necrotizing and crescentic glomerulonephritis, however, renal function often improves after immunosuppressive treatment, although relapses commonly occur.[30,59] As crescent formation is an ongoing process, as evidenced by the finding of crescents in various stages of development in renal biopsies, treatment might retard progression, even in well established disease. Furthermore, as noted earlier, fibrin deposits and even cellular crescents can sometimes disappear rather than be transformed into irreversible scars. It is clear, however, that crescents are not the only component of glomerular injury in conditions classified as crescentic glomerulonephritis. In particular, glomerular tuft necrosis and sclerosis as well as tubulointerstitial damage are often prominent.[56] Nevertheless, it is possible that measures that prevent macrophage accumulation might ameliorate these processes as well as interfere with crescent formation.

Furthermore, the special hazard of lung hemorrhage faced by many patients with crescentic glomerulonephritis may contraindicate anticoagulation or fibrinolytic therapy. Patients with anti-GBM nephritis or pauci-immune necrotizing and crescentic glomerulonephritis often develop severe lung hemorrhage.[60] Therefore, unless measures can be developed that specifically target anticoagulant or fibrinolytic agents to glomeruli, intervention might be more hazardous than helpful.

The current methods of treatment for human crescentic glomerulonephritis, which rely principally on corticosteroid and cyclophosphamide, sometimes with plasma exchange (for anti-GBM nephritis), are only partially effective and are attended by serious risks.[30,59] Several treatment protocols employing anticoagulants (often combined with anti-platelet agents, steroids, and immunosuppression) have been tried in various forms of renal disease with little or no evidence of beneficial effects.[61,62] It is of interest, however, that plasma exchange, which appears to be useful in anti-GBM nephritis,[30] may act to remove not only circulating anti-GBM antibodies but fibrinogen and clotting factors as well.[62] Encouraging results of anticoagulant and anti-platelet therapy have been obtained in some conditions not usually characterized by extensive crescents but in which endothelial damage, platelets, and intravascular coagulation may be important, as in the hemolytic uremic syndrome or lupus nephritis.[62,63] However, because the major forms of human crescentic glomerulonephritis are uncommon, it has not been possible to obtain adequate numbers of such patients for well controlled therapeutic trials.

Despite all of these problems, it is possible that safe and effective measures to prevent or remove glomerular fibrin deposits might still be shown to have some beneficial effects in rapidly progressive glomerulonephritis when combined with currently used immunosuppressive therapy. Even short-term therapy might prevent irreversible nephron loss resulting from extensive crescent formation. Thus, immunogenic agents, such as ancrod or heterologous antibodies against tissue factor or migration inhibitory factor, the effectiveness of which would be limited by an immune response against them, might have a place in therapy. Perhaps other nonimmunogenic antagonists of key mediators will be developed. Ultimately, specific suppression of the pathogenic immune response (as, for example, through blockage or depletion of antigen-specific T cells)[30,64] may provide the ideal therapy for immunologically mediated diseases, including renal diseases, but probably only in the distant future. For the present, interference with key mediators appears to be a more promising approach.

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Molecular Insights Into Renal Interstitial Fibrosis[1]

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ABSTRACT

Progressive interstitial fibrosis accompanied by loss of renal tubules and interstitial capillaries typifies all progressive renal diseases. Dynamic and complex, the process evidently overlaps with matrix remodeling; it may even be reversible. The interstitial fibrous tissue comprises several normal and novel matrix proteins, proteoglycans, and glycoproteins. Interstitial myofibroblasts are a major site of matrix protein overproduction, although resident fibroblasts, tubular cells, and inflammatory cells may contribute. Inadequate matrix degradation also appears to contribute to the fibrogenic process. Two protease cascades, the metalloproteinases and the plasminogen activator/plasmin family of serine proteases, are implicated in the turnover of interstitial matrix proteins; upregulated expression of protease inhibitors has been observed in each. Increased tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1 levels suggest that the intrinsic renal activity of the metalloproteinases and serine proteases are inhibited while matrix proteins accumulate in the interstitium. Several signals that may direct the interstitial fibrogenic process have been identified, but not yet proved to cause it. Upregulated expression of transforming growth factor β-1, the proteotypic fibrogenic cytokine, has been observed in experimental and human models; it probably does not act alone. There may be supportive roles for platelet-derived growth factor, interleukin-1, basic fibroblast growth factor, angiotensin II, and endothelin-1. Although it is not known why interstitial fibrosis compromises renal function, atrophy of renal tubules may be pivotal. Ischemic necrosis and/or apoptosis may generate nonfunctioning atubular and sclerotic glomeruli. Future studies must delineate the molecular basis of the differences between renal repair and renal destruction by fibrosis, two processes that share many common features.

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Landmark studies by Risdon *et al.* (1) and Schainuck *et al.* (2) were the first to highlight the fact that the severity of chronic tubulointerstitial disease is the single best histologic correlate of the decline in renal function and long-term prognosis. A quarter of a century later, investigators are still reaffirming the importance of chronic damage to tubules and the surrounding interstitial space to renal failure progression (3,4). Current thinking is that the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis share some pathogenetic mechanisms, but that these processes are not identical. Before reviewing the current knowledge of renal interstitial fibrogenesis, it is worthwhile to review Koch's postulates (5) and to emphasize that none of the candidate fibrogenic molecules has met these four criteria: (*1*) it produces a fibrogenic effect *in vitro*; (*2*) it is expressed *in vivo* in experimental models and human diseases with renal fibrosis; (*3*) inhibition of the molecule prevents or attenuates renal interstitial fibrosis; and (*4*) injection of the molecule into a normal host has a fibrogenic effect in the renal interstitium.

COMPOSITION AND ORIGIN OF THE INTERSTITIAL SCAR

The Interstitial Matrix

In the simplest terms, interstitial fibrosis is the accumulation of matrix proteins in the renal interstitium. An integral feature of interstitial fibrosis is the effect that it has on tubulointerstitial cells: The tubules and peritubular capillaries ultimately disappear; fibroblasts transform and increase in number; and mononuclear cells infiltrate into the interstitium (Figure 1). It is becoming apparent that the composition of the interstitial "scar" is complex (Table 1), with accumulation of both normal interstitial matrix proteins (collagens I, III, V, VII; fibronectin; tenascin) and unique ones such as matrix proteins normally restricted to tubular basement membranes (collagen IV, laminin). Fibronectin usually appears first. This adhesive glycoprotein is thought to form a scaffold for the deposition of other proteins; it also functions as a fibroblast chemoattractant (6).

Qualitative changes in the molecular composition of matrix proteins may be an important feature of the fibrogenic process. For example, the splicing patterns of fibronectin changed in experimental models of anti-glomerular basement membrane (anti-GBM) nephritis, anti-tubular basement membrane (anti-TBM) ne-

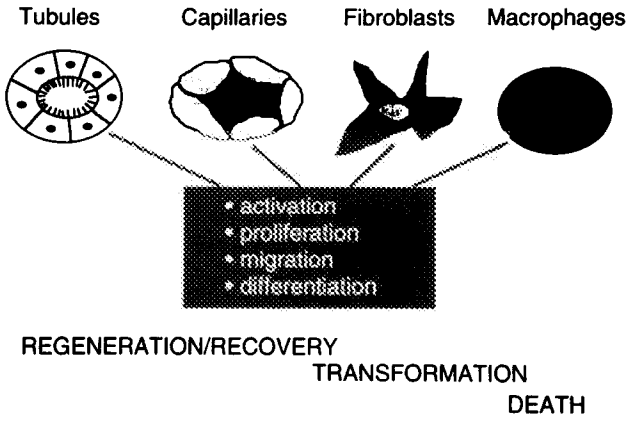

Figure 1. Summary of the cellular pathways that participate in renal interstitial fibrosis.

TABLE 1. Proteins that accumulate in the matrix in renal interstitial fibrosis

Interstitial Matrix Proteins
• Collagen I, III, V, VII
• Fibronectin
• Tenascin
Basement Membrane Proteins
• Collagen IV
• Laminin
Extracellular Proteoglycans[a]
• Large chondroitin sulfate proteoglycans (aggrecan, versican)
• Small proteoglycans (decorin, fibromodulin, biglycan)
• Basement membrane proteoglycans (heparin sulfate proteoglycan, perlecan)
Polysaccharides and Glycoproteins[a]
• Hyaluronan
• Thrombospondin
• Secreted protein, acidic and rich in cysteine (SPARC)

[a] Studies on the accumulation of extracellular proteoglycans, polysaccharides, and glycoproteins are in their infancy.

phritis (7), chronic graft-*versus*-host disease, and chronic serum sickness (8). Novel laminin epitopes that are found in normal developing but not mature glomeruli have been found to be re-expressed in glomeruli in advanced stages of graft-*versus*-host disease, a model of lupus nephritis (9). These molecular changes may occur independent of changes in the rate of matrix protein synthesis. Viedt and associates (10) reported that human tubular epithelial cells co-cultured with TGF-β1 synthesized increased quantities of fibronectin, but that most of the newly synthesized protein was the alternatively spliced fibronectin variant. Although the biologic significance of this switch is unknown, these authors speculate that the splice variant of fibronectin may have distinct functions.

The study of the renal interstitial accumulation of proteoglycans, glycoproteins, and polysaccharides is in its infancy (11). This class of molecules may modulate the function of cells and cytokines; it will be interesting to discover their role in the fibrogenic cascade (12). Hyaluronan (13), thrombospondin (14,15), and SPARC (secreted protein, acidic and rich in cysteine) (16,17) are examples of polysaccharides and glycoproteins recently shown to accumulate in the interstitium during progressive renal disease. An important issue needing investigation is whether each of these molecules is detrimental to renal function. Some of them may actually be beneficial, as suggested by the recent work of Nakao and coworkers (18) demonstrating that anti-GBM nephritis is more severe in tenascin-deficient mice.

Fibroblasts

To determine the cellular origin of the matrix proteins that accumulate in the renal interstitium, most studies have focused on the production of "traditional" interstitial proteins such as collagens I and III and fibronectin. In rat models of interstitial fibrosis induced by puromycin aminonucleoside (PAN) or adriamycin, increased renal mRNA levels and rates of matrix synthesis support the view that increased synthesis contributes to the fibrogenic process (19–21) (Figure 2). Assessment of collagen I gene expression in early kidney biopsies predicts the risk of renal fibrosis in a rabbit model of crescentic nephritis (22).

Which cells synthesize these proteins is not entirely clear. The limited *in situ* hybridization studies that have been performed to date have highlighted interstitial cells (20,23). It appears that cells from the fibroblast lineage are most important. Unfortunately, *in vivo* studies of interstitial fibroblasts have been hampered by the lack of good cellular markers.

It is presently unclear whether fibroblast proliferation is a necessary component of the fibrogenic process. In a rat model of chronic PAN nephrosis, my colleagues and I (19) counted the number of interstitial fibroblasts after immunostaining with ST3, a monoclonal antibody raised against rat bone-marrow fibroblasts that reacts with a subset of interstitial fibroblasts. We observed a modest increase in the number of ST3-positive fibroblasts over 13 wk (Figure 3). However, because fibroblasts derived from scarred kidneys show an increased rate of spontaneous proliferation *in vitro* compared with normal kidney fibroblasts (24), it seems reasonable to anticipate fibroblast proliferation as a component of the fibrogenic response.

Several fibroblast mitogens have been identified *in vitro*. A partial list includes interleukin-1, tumor necrosis factors α and β (dose-dependent), transforming growth factor β (conflicting data), platelet-derived growth factor, fibroblast growth factor, epidermal growth factor/transforming growth factor α, insulin growth factor-1, interferon α (25,26), plasminogen activator (27), fibrinogen (28), and endothelin-1 (29). However, very little is known about the mitogenic stimuli for renal fibroblasts *in vivo*. Recent studies by

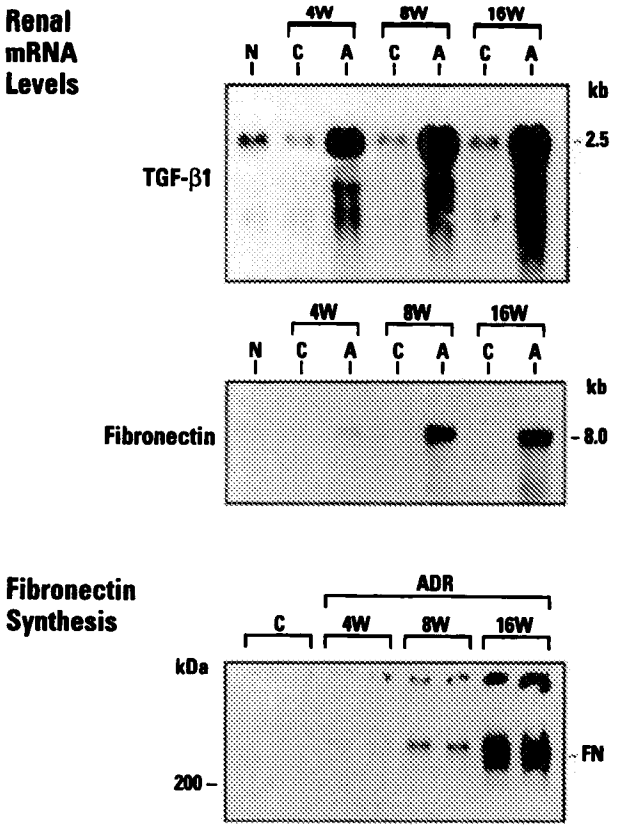

Figure 2. Increased renal cortical expression of transforming growth factor β-1 mRNA and fibronectin mRNA and synthesis in rats with adriamycin nephropathy. The upper panel illustrates the increase in TGF-β1 mRNA levels in adriamycin-treated (A) compared to control (C) rats at 4, 8, and 16 wk. The middle panel shows the increase in renal cortical fibronectin mRNA levels. The lower panel illustrates the corresponding increase in renal cortical fibronectin synthesis as obtained by immunoprecipitation of ^{35}S-methionine-labeled cortical conditioned media prepared from control (C) and adriamycin (ADR)-treated rats. This figure is reproduced from work by Tamaki *et al.* (21) with copyright permission from Blackwell Science Inc.

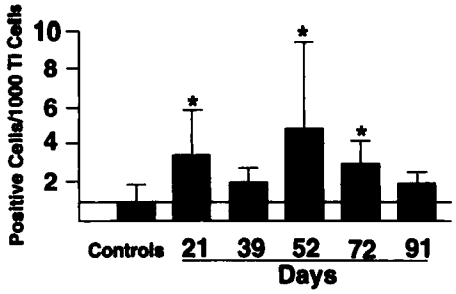

Figure 3. The number of ST3-positive interstitial fibroblasts in rats with chronic puromycin aminonucleoside nephrosis. Results represent the mean ± 1 SD. * $P < 0.05$, Bonferroni's *t* test. TI, tubulointerstitial. This figure is reproduced in part from Jones *et al.* (19) with copyright permission from Blackwell Science Inc.

Lonnemann *et al.* (30) provide some early clues that mitogens for renal fibroblasts may differ if the fibroblasts are in a normal compared with a fibrotic milieu. For example, fibrosis-derived renal fibroblasts proliferated in response to interleukin-1β (IL-1β), whereas proliferation of normal kidney fibroblasts was inhibited by IL-1β. In contrast to skin fibroblasts, kidney fibroblasts derived from both normal and fibrotic kidneys failed to proliferate following exposure to the classic fibroblast mitogen, basic fibroblast growth factor (30,31).

The plasticity of the phenotype of resident fibroblasts is becoming increasingly clear. It is possible that alterations in the phenotype and "behavior" of pre-existing renal fibroblasts may be one of the most important events associated with increased matrix synthesis. Müller and his colleagues (24,32) have subdivided renal fibroblasts into six phenotypic groups that differ in their mitotic and matrix-synthesizing activities, with the last three groups ascribed to "postmitotic fibroblasts." A better understanding of this kind of behavioral alteration should help to clarify the cellular basis of renal fibrosis. For example, Rodemann and Müller's studies (33) have shown that fibroblasts derived from fibrotic human kidneys synthesize more total collagen than those derived from normal kidneys. This is true whether the fibroblasts are in a mitotic or postmitotic phase.

The ability of interstitial fibroblasts to assume a myofibroblastic phenotype, presumably representing a state of activation, has recently been reported in human (34,35) and animal models (36–40) of progressive renal disease. With immunostaining, the myofibroblasts can be identified as interstitial cells expressing α smooth-muscle actin (Figure 4). Transforming growth factor β-1 (TGF-β1) (41) and platelet-derived growth factor B (PDGF-B) (42) are two growth factors that can transform fibroblasts to myofibroblasts. The origin of these interstitial "myofibroblasts" is not entirely known. They may derive exclusively from traditional interstitial fibroblasts; however, there is increasing evidence that some of these cells are perivascular cells that have migrated into the interstitium. In a rat model of anti-GBM nephritis, Wiggins and associates (43) showed that the earliest increases in procollagen α1(I) mRNA transcripts occurred in perivascular cells. Studies by Ronnov-Jessen *et al.* (44) using a human breast-cancer explant model suggest that myofibroblasts come from venules. Their studies demonstrated that, with migration of these cells into the interstitium, capillary obliteration (a common feature of progressive renal interstitial fibrosis) may ensue.

Macrophages

At present we should not dismiss the possibility that interstitial macrophages also contribute to the expending interstitial pool of matrix proteins. Cultured

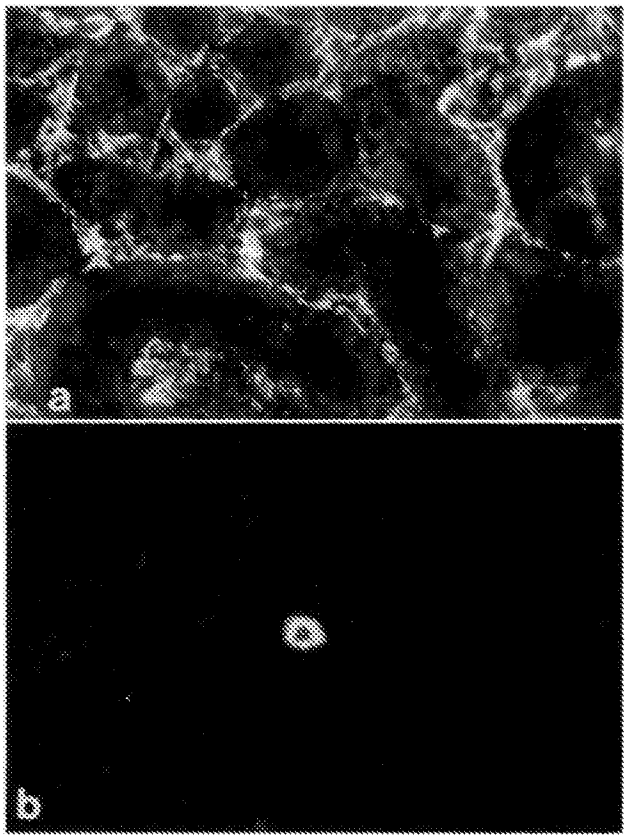

Figure 4. Immunofluorescence photomicrograph illustrating the appearance of α smooth-muscle actin-positive interstitial myofibroblasts in a rat with acute puromycin aminonucleoside nephrosis (a). In normal rats, α smooth-muscle actin-positive cells are restricted to the walls of arteries (b). (a, b, original magnification ×350)

macrophages have been shown to synthesize collagen I and fibronectin (45). An interstitial infiltrate of macrophages is present in all kidneys with progressive renal disease (46,47). When the renal interstitium becomes inflamed, circulating monocytes migrate into the interstitium under the direction of specific chemotactic stimuli and adhesion molecules that largely remain to be identified. Resident interstitial macrophages may also proliferate *in situ*, as recently shown by Lan and colleagues (48,49) in a rat model of Goodpasture's syndrome.

Tubular Cells

The contribution made by tubular cells to the expanding interstitial matrix pool is not yet clear. Normally a source of tubular basement-membrane proteins, it is of interest whether tubular cells produce the basement membrane proteins that appear *de novo* in the interstitium during renal fibrosis. Tubular cells overproduce collagen IV in experimental diabetes, a change that can be revised by low-molecular-weight heparin (50). It has been proposed that tubular cells behave differently after exposure to fibrogenic cytokines. Using kidney epithelial cells from a line of normal rats, Creely *et al.* (51) observed an eightfold increase in collagen I production after exposure to TGF-β1; the rate of collagen IV synthesis was unchanged.

It is also possible that the remodeling of the interstitial architecture during fibrosis results in the migration of tubular epithelial cells into the renal interstitium. In a study of human end-stage kidneys, Nadasdy *et al.* (52) identified cells within the interstitium that expressed an epithelial membrane antigen. Recently, Strutz and associates (53) reported a process of tubular transdifferentiation in murine models of anti-basement membrane disease, whereby tubular epithelial cells are induced to express fibroblast-specific proteins. These investigators have proposed the novel hypothesis that epithelial cells may be converted into fibroblasts at the site of injury. The transforming signals remain to be elucidated.

MATRIX TURNOVER

There is reasonable evidence that interstitial fibrosis is not exclusively caused by an increase in the rate of matrix synthesis but that impaired degradation of matrix proteins is also involved. In models of fibrosis caused by ligation of the renal vein or one ureter, González-Avila and coworkers (54) failed to find increased collagen synthesis rates, but did observe impressive reductions in renal collagenolytic activity to one-thirtieth and one-tenth of baseline, respectively (Table 2). The existence of an active intrarenal matrix-degrading enzyme cascade is also supported by our studies (20) of rats with acute PAN nephrosis. In that model, the early foci of interstitial fibrosis evident at 3 wk actually disappeared by 6 wk, suggesting that early interstitial fibrosis may be reversible before the formation of an organized "scar." The concept of reversible matrix deposition is supported by a study (55) in a rat model of acute tubular necrosis showing transient accumulation of fibronectin.

There are four families of connective tissue proteases. Two of these, the metalloproteinases and the serine proteases, are of current interest with respect to their possible role in renal fibrosis.

The Metalloproteinase Family

The metalloproteinases (Figure 5) are a large group of enzymes subdivided into three families—the inter-

TABLE 2. Collagen turnover in renal fibrosis[a]

Type of Collagen Turnover	Renal Vein Ligation	Ureteral Obstruction
Collagen Concentration	5 × ↑	3 × ↑
Collagen Synthesis	5 × ↓	No change
Collagenolytic Activity	30 × ↓	10 × ↓

[a] This data is based on González-Avila *et al.* (54).

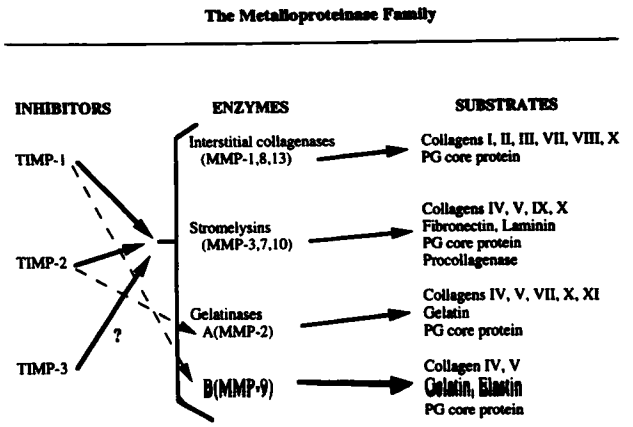

Figure 5. The classic metalloproteinase family members and its inhibitors. MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; PG, proteoglycan.

stitial collagenases, stromelysins, and gelatinases—that together are capable of degrading virtually all matrix proteins (56,57). The three tissue inhibitors of metalloproteinases, TIMP-1, -2, and -3, are enzyme inhibitors that may inhibit several members of this protease family.

To date, the inhibitor most extensively studied in renal interstitial fibrosis is TIMP-1. TIMP-1 inhibits all of the latent prometalloproteinases and is emerging as a likely participant in renal fibrosis. Renal TIMP-1 mRNA levels are significantly elevated in several experimental models of interstitial fibrosis, including protein-overload proteinuria (2.3× control levels) (58), hypercholesterolemia-induced renal disease (2.7×) (59), passive Heymann nephritis (3.3×) (60), PAN nephrosis (11×) (19,20,61) (Figure 6a), anti-TBM nephritis (11×), obstructive uropathy (13.7×) (62,63), and diabetic nephropathy (64). These findings predict a decrease in the rate of degradation of matrix proteins within the kidney while matrix proteins accumulate in the interstitium. Further studies are necessary to confirm this prediction.

TIMP-1 protein is not detected by immunostaining in normal rat kidneys. In all of the models that we have studied, TIMP-1 appears in the interstitium during the course of renal fibrosis. With *in situ* hybridization techniques, we have localized TIMP-1 transcripts to both interstitial cells and tubular epithelial cells in rats with protein-overload proteinuria (65). Using cultured cells, Norman and colleagues (66) demonstrated TIMP production by normal tubular cells and interstitial fibroblasts, which is increased when these cells are isolated from humans with autosomal-dominant polycystic kidney disease. In the model of polycystic kidney disease in the Han: Sprague-Dawley (SPRD) rat, Schaefer and associates (67) also found increased renal tubular mRNA levels for TIMP-1 and TIMP-2. A positive correlation between the presence of myofibroblasts and overexpression of TIMP-1 has been reported (68).

Further studies are needed to clarify whether

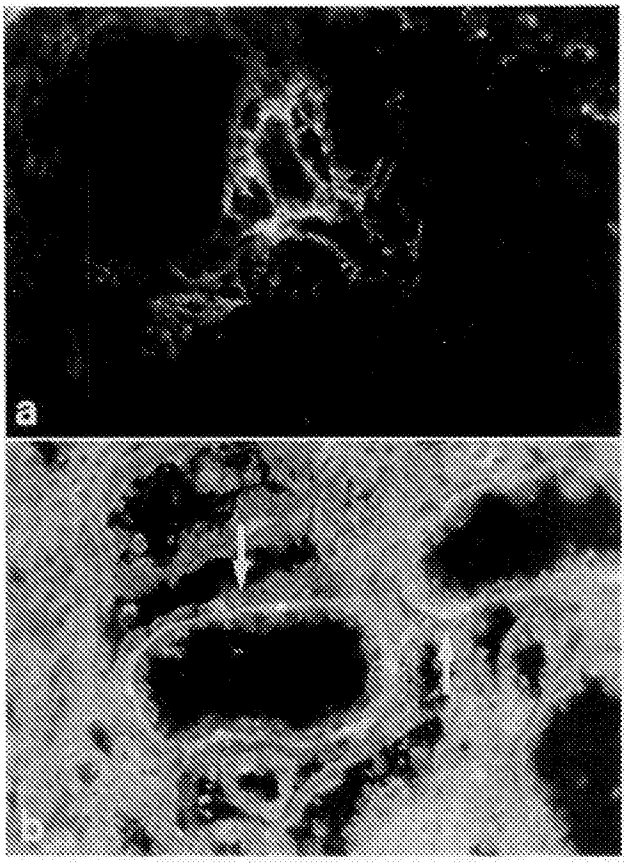

Figure 6. Immunofluorescence photomicrographs illustrating the *de novo* expression of protease inhibitors in experimental models of interstitial fibrosis (a). Interstitial expression of the tissue inhibitor of metalloproteinases and (b) tubular expression of plasminogen activator inhibitor-1 (arrows) in a rat with acute puromycin aminonucleoside nephrosis. (a, original magnification ×350; b, original magnification ×270)

changes in the levels of the metalloproteinases also contribute to renal fibrosis. Recent *in vitro* studies (69) confirm that tubular cells and interstitial fibroblasts are a source of metalloproteinases, although the specific enzymes that are expressed show regional heterogeneity, dependent in part on the matrix composition of the substrate upon which they are cultured. Tubular cells may secrete matrix metalloproteinases both apically and basolaterally. We have not detected statistically significant changes in renal mRNA levels for interstitial collagenase or stromelysin in any of the experimental models of renal fibrosis that we have studied. Renal mRNA levels for the 72-kd Type IV collagenase, MMP-2 or gelatinase, were transiently increased in a rabbit model of obstructive uropathy (62), whereas renal tubular mRNA and enzyme activity levels have been reported (67) to be significantly decreased in rat polycystic kidney disease.

Results of a study (58) in rats with protein-overload proteinuria highlight upregulated expression of TIMP-1 as a significant mediator of interstitial fibrosis.

We have developed this model by injecting uninephrectomized rats with large daily intraperitoneal doses of BSA. The total kidney collagen is an impressive 200% of control levels by 3 wk. This degree of fibrosis would not have been predicted by examining matrix-protein mRNA levels. For example, procollagen III levels did not differ significantly from that of control kidneys, but it nevertheless was shown to accumulate in the interstitium. Renal TIMP-1 mRNA levels were significantly increased, to 260% of control kidney levels.

We have also developed a murine model of BSA-induced overload proteinuria and confirmed that renal expression of TIMP-1 is also increased (unpublished). TIMP-1 knockout mice that express a mutant form of TIMP-1 develop less severe interstitial fibrosis than wild-type mice, but fibrosis is not completely eliminated (AA Eddy, unpublished data). We observed a similar renal mRNA profile in uninephrectomized rats with hypercholesterolemia (59): Despite very modest changes in renal matrix-protein mRNA levels, after 12 wk of diet-induced hypercholesterolemia, the rats developed interstitial fibrosis with a 30 to 50% increase in total kidney collagen. Renal TIMP-1 mRNA levels were significantly increased at all examined time points (4, 8, and 12 wk).

The Plasma-Dependent Pathway

In addition to the metalloproteinase family, the second proteolytic cascade that may play a role in renal interstitial fibrosis is the plasmin-dependent pathway (70,71) (Figure 7). Plasmin not only activates latent procollagenases but also may directly degrade some matrix proteins such as fibronectin and laminin. Latent plasminogen is activated by proteolytic cleavage by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). These enzymes are inhibited by plasminogen activator inhibitors (PAI). As with the metalloproteinase family, over-

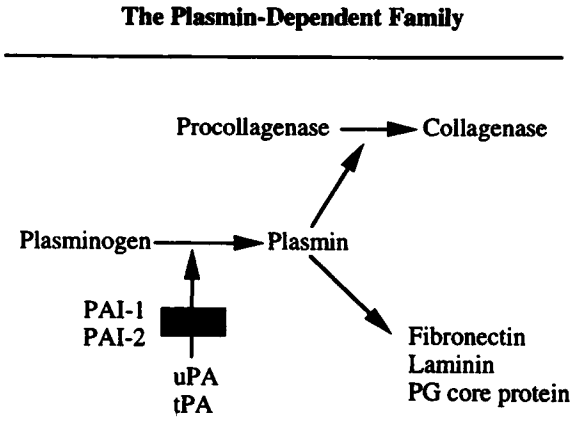

Figure 7. The plasmin-dependent pathway of proteases and their inhibitors. PAI, plasminogen activator inhibitor; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; PG, proteoglycan.

expression of an enzyme inhibitor, in this case PAI-1, is emerging as a common theme in progressive renal disease. We have found significant elevations in renal PAI-1 mRNA levels in rats with PAN nephrosis (3.2× control levels; unpublished) and protein-overload proteinuria (2.0×) associated with increased tubular expression of PAI-1 protein (58) (Figure 6b). An *in situ* hybridization study (65) also highlights tubular cells as the source of increased PAI-1.

To date our studies have failed to detect significant changes at the mRNA level for any of the proteolytic enzymes, with one exception: downregulated expression of uPA in the rat model of diet-induced hypercholesterolemia (59). In this model, interstitial fibrosis develops very slowly. By 12 wk, total kidney collagen contents were increased to 130 to 150% of control levels. Rather surprisingly in this model, renal matrix protein mRNA levels were not increased compared to those in control kidneys, except for a modest 30% increase in procollagen I and a 70% increase in procollagen III at 12 wk. Although PAI-1 mRNA levels were not significantly different, uPA mRNA levels were significantly decreased to 40% of control levels throughout the study period (4, 8, and 12 wk). However, until a role for the plasmin-dependent pathway is confirmed by inhibition studies or by studies in deficient mice, these results must be interpreted cautiously. Plasmin is one of the few known activators of latent TGF-β1 (72); downregulation of plasmin activity may therefore also have anti-fibrogenic effects. However, it is noteworthy that bleomycin-induced pulmonary fibrosis is more severe in PAI-1–overexpressing mice and attenuated in PAI-1–deficient mice (73).

THE FIBROGENIC SIGNALS

Although several cytokines have fibrogenic potential (25,26,74) (Table 3), TGF-β1 remains the premier fibrogenic cytokine and the only one that has been studied in any detail in renal interstitial disease. Yet, despite a great deal of circumstantial evidence, definitive proof that TGF-β1 plays a role is still not available. Active TGF-β1 elicits a variety of responses that are relevant to renal fibrosis, including increased matrix synthesis, inhibition of matrix degradation, upregulation of the integrin matrix-adhesion molecules, and chemoattraction of fibroblasts and monocytes (75,76). The net effect of TGF-β1 is to tip the

TABLE 3. Fibrogenic cytokines[a]

Fibroblast Growth Factor
• Interleukin-1 α
• Interleukin-1 β
• Platelet-derived growth factor
• Transforming growth factor α
• Transforming growth factor β
• Tumor necrosis factor α

[a] This table is based on data of Kovacs and DiPietro (25) and Korthagen *et al.* (26).

balance of matrix synthesis and degradation, toward the accumulation of matrix proteins. Several laboratories, including our own, have been interested in the role of TGF-β1 in renal interstitial fibrosis (Table 4). In four experimental models that we have studied, renal TGF-β1 mRNA levels have been increased to 200% to 400% of control levels (58–61). By *in situ* hybridization and immunostaining techniques, interstitial cells and tubules appear to be the source of the increase in TGF-β1 production (Figure 8). Our studies of rats with PAN-induced nephrosis suggest that interstitial macrophages are an important source of TGF-β1 (61), although fibroblasts (77) and myofibroblasts (78) have been known to be the major TGF-β1-producing interstitial cells in other experimental models. Nephrotic rats fed a low-protein diet have less proteinuria and fewer interstitial macrophages than nephrotic rats fed standard chow (61). The twofold increase above control levels in renal TGF-β1 mRNA levels observed in the nephrotic rats was normalized in the nephrotic rats fed a low-protein diet. This difference had an important biologic effect, leading to a significant reduction in total kidney collagen.

The importance of tubular cells as a source of increased TGF-β1 has not been extensively investigated *in vivo*. We found a twofold increase in TGF-β1 mRNA levels in proximal tubules isolated from rats with acute puromycin aminonucleoside nephrosis, above those in normal rat kidneys (61). There is conflicting evidence that interstitial cells (79) and the tubules (80) are a major source of TGF-β1 in a rat model of obstructive nephropathy. After acute ischemic renal injury, regenerating tubules express TGF-β1 (81). Increased tubulointerstitial expression of TGF-β1 correlates with interstitial accumulation of an alternatively spliced isoform of fibronectin (80) and PAI-1 in a variety of chronic glomerular diseases in humans (82) and in human renal allografts with chronic rejection (83–85).

There are multiple levels of control of TGF-β1 that also should be considered before increased bioactivity can be assumed in these *in vivo* studies. Newly synthesized TGF-β1 must be activated by proteolytic cleavage of the N-terminal peptide, latency-associated peptide (LAP) (86). This important activation step has received little attention *in vivo*. It may occur after exposure of latent TGF-β1 to acid pH (87); by proteolytic cleavage accomplished by enzymes such as plasmin, cathepsin D, furin, or glycosidases (72,88,89); and after binding of the latent complex to the glyco-

TABLE 4. Reported occurrences of increased TGF-β1 expression in renal interstitial fibrosis

Disorder	Reference(s)
Animal Studies	
Puromycin aminonucleoside nephrosis	19, 20, 61
Adriamycin nephropathy	21, 92
Protein-overload proteinuria	58
Diet-induced hypercholesterolemia	59
Passive Heymann nephritis	60
Anti-GBM nephritis	147
Chronic anti-Thy-1 nephritis	78
Immune complex nephritis	106
Murine lupus nephritis	148
HIV nephropathy	149, 150
Diabetic nephropathy	151, 152
Obstructive uropathy	79, 80, 109, 153
Polycystic kidney disease	154
Cyclosporine nephrotoxicity	77, 155
Acute tubular necrosis	81, 156
TGF-β1-overproducing mice	100, 101
Human Studies	
IgA nephropathy	157
Chronic glomerulonephritis	82, 158
Diabetic nephropathy	152
Chronic renal allograft rejection	83–85

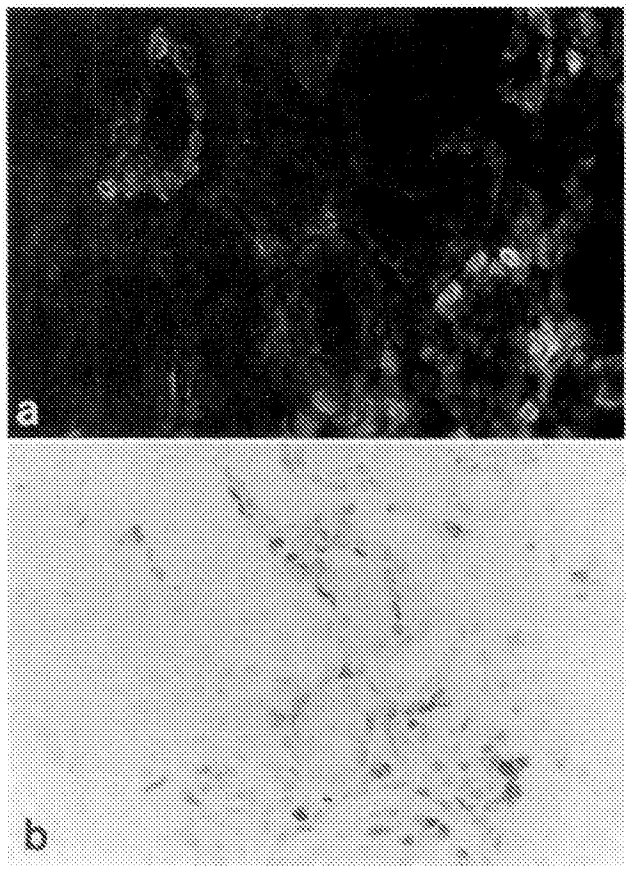

Figure 8. Photomicrographs showing the increased expression of TGF-β1 in experimental models of interstitial fibrosis. (a) Immunostaining with an antibody to the bioactive TGF-β1 peptide illustrates positive tubular and interstitial cells in a rat with bovine serum albumin-induced overload proteinuria. (b) *In situ* hybridization using a rat TGF-β1 antisense riboprobe highlights positive interstitial cells in a rat with protein-overload proteinuria. (a, original magnification ×380; b, original magnification ×300)

protein thrombospondin (90). LAP may subsequently bind noncovalently to and inactivate TGF-β1 (86). A second gene product, latent TGF-β–binding protein, also alters the bioavailability of TGF-β1. Not detectable in renal tubules of normal rats (91), TGF-β–binding protein has been shown to accumulate in the interstitium of rats with adriamycin nephrosis (92).

However, the story does not end there. TGF-β1 is a member of the crinopectins, a term introduced by Feige and Baird (93) to refer to molecules secreted by cells that subsequently adhere to specific pericellular structures, where they are sequestered under a latent form. Several potential extracellular storage sites exist for TGF-β1, including small proteoglycans such as decorin, biglycan, betaglycan, fibromodulin, and endoglin. The most important inhibitor of TGF-β1 in plasma, α_2-macroglobulin, may also accumulate in the kidney during progressive disease (94). TGF-β receptors are ubiquitous (86), and little is currently known about alterations in receptor expression in interstitial renal disease. Tamaki *et al.* (21) reported a progressive increase in renal mRNA levels for receptors II and III during the course of adriamycin nephropathy.

So, does TGF-β1 cause interstitial fibrosis? Border and colleagues (95) have inhibited TGF-β1 with a neutralizing antiserum or by using the neutralizing proteoglycan decorin (96,97) to establish a role for TGF-β1 in the transient glomerular sclerosis that follows the injection of rats with anti-Thy-1 antiserum. Unfortunately, similar inhibition studies in animals with progressive interstitial fibrosis still have to be done. The task has proven challenging, in part because prolonged systemic depletion of TGF-β1 is fatal, at least in mice (98,99), making it necessary to develop neutralization or inhibition strategies specifically targeted to the kidney. However, it is worth noting that TGF-β1 transgenic mice with high plasma levels of active TGF-β1 do develop chronic tubulointerstitial disease after the onset of glomerulosclerosis (100,101).

There is some evidence to suggest that TGF-β1 alone might not induce interstitial fibrosis in a normal kidney. Terrell and colleagues (102) administered human recombinant TGF-β1 chronically to rats and rabbits and observed glomerulosclerosis while the renal interstitium remained unaffected (T. Terrell, Genentech Inc., San Francisco, CA, personal communication). However, all progressive renal diseases are characterized by an interstitial infiltrate of mononuclear cells that may establish a permissive fibrogenic environment; these cells are capable of releasing a myriad of products. For example, a recent study (103) of renal biopsies obtained from patients with IgA nephropathy showed a nice correlation between the cytokine profile of interstitial cells (IL-1, IL-6, IL-8, TNF-α) and the histologic severity of tubulointerstitial disease.

Little is currently known about *in vivo* stimulation of TGF-β1 synthesis, but one fascinating scenario is evolving and was recently reviewed by Egido (104). Using cultured renal tubular cells, Wolf and coworkers (105) observed that angiotensin II stimulates TGF-β1 production in renal tubular cells. More recently, angiotensin II has been reported (106) to upregulate TGF-β1 expression in fibroblasts. Angiotensin-converting enzyme inhibitors or an angiotensin II receptor antagonist decreased TGF-β1 and collagen IV mRNA levels in a rat model of obstructive uropathy (107–109); these changes were associated with preservation of the renal interstitium. Rats treated with an angiotensin-converting enzyme inhibitor plus an angiotensin II receptor antagonist also develop less severe cyclosporine-induced interstitial fibrosis (110). Enalapril has been reported to attenuate interstitial fibrosis in rats with chronic aminonucleoside nephrosis (111), in the rat remnant-kidney model (112), and in aging mice (113). Unfortunately, changes in renal TGF-β1 expression were not evaluated in the later three studies. In normotensive rats with chronic immune complex nephritis, treatment with the angiotensin-converting enzyme inhibitor quinapril decreased renal cortical mRNA levels for TGF-β1, fibronectin, and collagens I, III, and IV. Tubulointerstitial damage was essentially prevented in the quinapril-treated group (114). Preliminary data suggests that angiotensin II may also directly regulate matrix gene expression (115).

However, it is presently premature to conclude that angiotensin II plays an essential role in renal fibrosis. A recent study (77) in a rat model of cyclosporine nephropathy reported that treatment with either an angiotensin II type I receptor antagonist or hydralazine plus furosemide reduced TGF-β1 expression and the severity of interstitial fibrosis. The angiotensin II story has been further complicated by the observation (116) that mice with a homozygous null mutation in the angiotensinogen gene by 3 wk of age develop histologic evidence of mild interstitial fibrosis associated with an increase in renal cortical TGF-β1 mRNA. It is possible that the concurrent upregulation of PDGF-β that was observed in these mice may mediate the increased expression of TGF-β1. However, this study suggests that angiotensin II is not essential for upregulated renal expression of TGF-β1.

Very little data is available about the role of other fibrogenic cytokines in renal fibrosis. Although TGF-β1 is considered to be the premier fibrosis-promoting cytokine, PDGF is thought to have significant fibrogenic effects (117), whereas several other cytokines have been ascribed functions that may facilitate fibrosis. These include fibroblast growth factor, interleukin-1, tumor necrosis factor α, and transforming growth factor α (25,74) (Table 3). Treatment of rats with recombinant PDGF-BB induced tubulointerstitial cell proliferation, the appearance of interstitial myofibroblasts, and subsequent interstitial fibrosis (42). Renal TGF-β1 mRNA levels were not increased in the PDGF-BB–treated rats. Increased interstitial cell expression of the β receptor for PDGF has been observed in diseased human kidneys (118,119). In-

creased expression of PDGF B-chain and the PDGF receptor B-subunit by tubules and interstitial cells has been reported in rats with progressive renal disease after five-sixths nephrectomy (36). Increased PDGF B-chain expression has been reported in regions of tubulointerstitial injury induced by the continuous infusion of angiotensin II (37).

Interleukin-1 (IL-1) is a cytokine with important proinflammatory effects and glomerular production of IL-1 is increased in several types of experimental and human glomerulonephritis. IL-1 also stimulates fibroblast proliferation and possibly matrix protein synthesis (25,74,120). The presence of IL-1β–positive interstitial cells has been shown to correlate with the decline in renal function in a group of patients with primary glomerular disease (120), and the number of IL-1–positive interstitial cells correlates with the extent of interstitial damage in a group of patients with IgA nephropathy (103). In a rat model of crescentic glomerulonephritis, tubular atrophy and interstitial fibrosis were prevented by treatment with a soluble recombinant IL-1 receptor antagonist (121,122). However, it is likely that the ability of the IL-1 receptor antagonist to prevent interstitial fibrosis is indirect, related to its ability to abrogate acute renal injury and interstitial infiltration by leukocytes.

Morita and associates (123) recently reported the appearance of interstitial cells expressing fibroblast growth factor (FGF) in biopsy specimens from patients with chronic renal disease. Ichimura *et al.* (124) reported increased FGF expression by interstitial and tubular cells after a nephrotoxic insult to rats. In a murine model of HIV-associated nephropathy, basic FGF and its receptors co-localized with areas of interstitial extracellular matrix accumulation (125).

There is experimental evidence (104) that endothelin-1 has fibrosis-promoting effects. Endothelin-1 is expressed by proximal and distal tubules, cortical and medullary collecting ducts (126), fibroblasts (29,127), endothelial cells (128), and macrophages (129). Endothelin-1 has been reported to stimulate proliferation of fibroblasts including human renal fibroblasts (130) and to increase collagen synthesis and decrease collagenase activity in cardiac fibroblasts (131). It is presently unclear whether the profibrotic effects of endothelin are mediated directly or indirectly via the release of cytokines or other lipid and protein mediators (104). Renal preproendothelin-1 gene expression increases in rats after five-sixths nephrectomy and in murine lupus nephritis (132). Treatment of these animals with an antagonist to the endothelin receptor ET_A significantly reduced the severity of the tubulointerstitial damage (133,134). Overexposure of cultured tubular cells to various purified proteins, including albumin, stimulates endothelin-1 synthesis, suggesting a possible link between persistent proteinuria and chronic tubulointerstitial injury mediated via endothelin-1 (135). Transgenic mice that overexpress human endothelin-1 develop glomerulosclerosis (F. Theuring, F Schmager, C. Thöne-Reinicke, S. Chotzen, T. Quertermoust, W.-D. Schleuning, B. Hocher. Transgenic mice in endothelin research. Fourth International Conference on Endothelin, London, UK, 1995), and transgenic rats overexpressing human endothelin-2 develop severe interstitial fibrosis (M. Paul, G. Schönfelder, L. Liefeldt, W. Böcker, A. Lippoldt, M. Zintz. Renal phenotype in transgenic rats expressing the human endothelin-2 gene. Fourth International Conference on Endothelin, London, UK, 1995).

THE VICTIMS OF INTERSTITIAL FIBROSIS

The ultimate question to be answered is why interstitial fibrosis causes a decline in renal function. A significant factor is likely to be the ultimate obliteration of the renal tubules by the fibrogenic process. In fact, if you look back to the seminal study by Risdon *et al.* (1) (Figure 9), the degree of tubular atrophy showed a significant correlation with serum creatinine levels. Glomerular sclerosis is not necessarily an immediate consequence of tubular atrophy, as suggested by the studies of Marcussen (136) that demonstrated the presence of atubular glomeruli in the kidneys of patients with chronic renal function. Tubules are damaged early in the course of progressive renal disease. Several mechanisms of tubular injury have been proposed; and for each of them, proteinuria is an aggravating factor. These include damage resulting from tubular obstruction, lysosomal enzymes, reactive oxygen metabolites, complement proteins, and ischemia (137). In response to injury, tubular cells may alter their expression of a variety of cytokines and vasoactive peptides, as recently reviewed by Ong and Fine

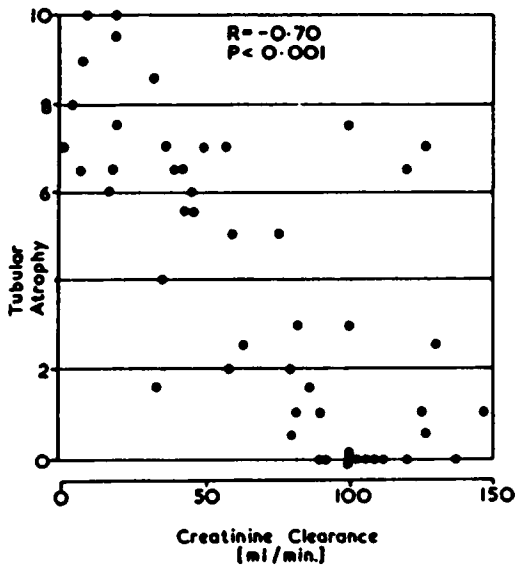

Figure 9. Correlation between the degree of tubular atrophy and the creatinine clearance in a group of patients with chronic glomerular disease. This work is reproduced from Risdon *et al.* (1) with copyright permission from The Lancet Ltd.

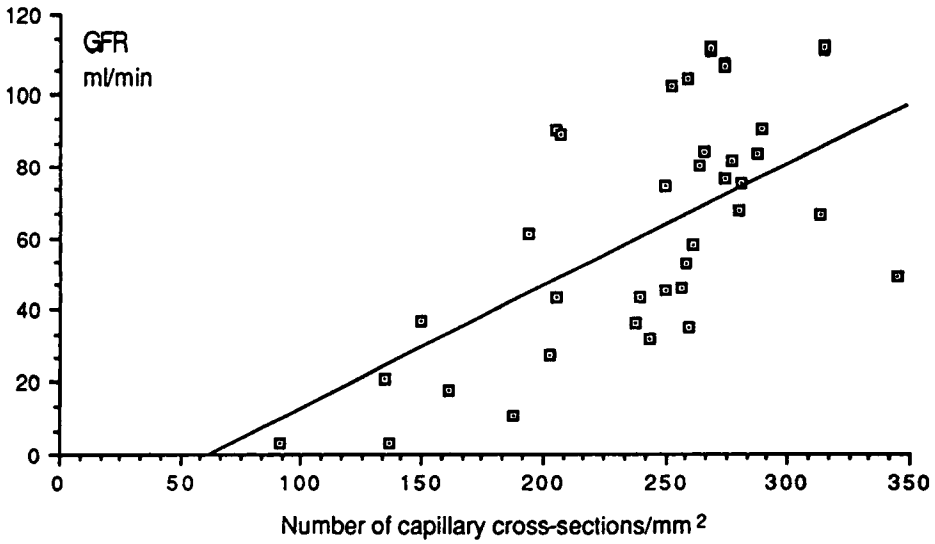

Figure 10. Correlation between the cross-sectional area of interstitial capillaries and the glomerular filtration rate in a group of patients with chronic renal disease ($r = 0.64$, $P < 0.0001$). The work was reproduced from Serón *et al.* (142) with copyright permission from Oxford University Press.

(138). Among our challenges is the determination of which of these changes preserve and which destroy tubular structures. Whether apoptosis or necrosis is the route of the tubular dropout that accompanies interstitial fibrosis also requires investigation. A significant increase in apoptotic tubular cells has been reported in a rat model of renal fibrosis caused by obstructive uropathy (139).

The intricate network of interstitial capillaries that percolate through the interstitial space is also obliterated during the course of interstitial fibrosis. A decrease in the peritubular capillary volume in areas of renal interstitial inflammation and fibrosis was first described by Ljungquist in 1963 (140). Bohle and associates (141) confirmed a significant correlation between the reduced area of postglomerular capillaries and the serum creatinine level. Using a monoclonal antibody that recognizes capillary endothelium to stain human kidney biopsies, Serón *et al.* (142) found a very strong correlation between the reduction in interstitial capillary area and the decline in GFR (Figure 10). Even before permanent loss of peritubular capillaries occurs, overexpression of vasoconstrictors such as adenosine, endothelin, angiotensin II, and PDGF may compromise the oxygen supply to the tubulointerstitium, especially during periods of high metabolic activity (143). Fine and colleagues have emphasized the role of systemic hypertension as a factor that may trigger the cascade that results in tubulointerstitial ischemia and ultimately fibrosis (126,143).

It is tempting to speculate that a relationship might exist between capillary loss and the disappearance of tubules mediated by ischemic injury. Chronic renal ischemia alone can cause interstitial inflammation, tubular atrophy, and interstitial fibrosis (144). Preliminary *in vitro* studies (145) suggest that hypoxia itself may have direct fibrogenic effects. Anoxia may also activate local macrophages (146).

In the final analysis it is the cells of the tubulointerstitium, resident, transformed, and infiltrating, that direct the fibrogenic cascade (Figure 1). A major challenge for the future will be to determine the pathways that differentiate wound healing to preserve *versus* fibrogenesis that destroys the tubulointerstitial architecture.

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